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Award Number: DAMD 17-03-1-0332

TITLE: Breast Cancer Suppression by IDO Inhibitors

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REPORT DATE: May 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-05-2006	Final	1 May 2003 – 30 Apr 2006

4. TITLE AND SUBTITLE

Breast Cancer Suppression by IDO Inhibitors

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0332

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

George C. Prendergast, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Lankenau Institute for Medical Research
Wynnewood, PA 19096

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

Immune escape is a crucial feature of cancer progression about which little is known. Elevation of the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) in tumor cells can facilitate immune escape. Not known is how IDO becomes elevated or whether IDO inhibitors will be useful for cancer treatment. Based on evidence that Bin1 loss elevates IDO and enhances immune evasion by tumor cells, we hypothesized that reversing IDO activity with chemical inhibitors would enhance immune recognition and rejection of tumor cells. We aimed to (1) generate an IDO antibody to assess its expression in normal and malignant breast tissue, (2) identify pharmacologically attractive 'lead' inhibitors of IDO, and (3) evaluate the ability of compounds with IDO inhibitory activity to block tumor growth in the MMTV-neu mouse model of breast cancer. In Year 3 of this project we report progress beyond the Aims as originally proposed, which were completed last year. Briefly, we report progress on new inhibitors and findings related to compound formulation, mechanism, and efficacy. This highly successful project has helped drive the efforts of a multidisplinary team of government, biotech, and academic groups who are collaborating to move a lead IDO inhibitor into Phase I clinical trials later this year.

15. SUBJECT TERMS
Breast Cancer

16. SECURITY CLASSIFICATION OF:				18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	64	19b. TELEPHONE NUMBER *(include area code)*

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Reprints provided:

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Muller, A.J. and Prendergast, G.C. (2005). Marrying immunotherapy with chemotherapy: why say IDO? Cancer Res. 65, 8065-8068.

Malachowski, W.B, Metz, R., Prendergast, G.C., and Muller, A.J. (2005). A new cancer immunosuppression target: indoleamine 2,3-dioxygenase (IDO). A review of the IDO mechanism, inhibition, and therapeutic applications. Drugs Fut. 30, 897-905.

Gaspari, P., Banerjee, T., Malachowski, W.P. Muller, A.J., Prendergast, G.C., DuHadaway, J., Bennett, S. and Donovan, A.M. (2006). Structure-activity study of brassinin derivatives as indoleamine 2,3-dioxygenase inhibitors. J. Med. Chem., 49, 684-692.

Introduction

Immune escape is a fundamental trait of cancers including breast cancer. While there is great interest in ways to stimulate immune rejection of breast cancers, no effective strategies to achieve this end have been identified. Such strategies are particularly appealing for the treatment of systemic disease, which constitutes the major clinical challenge. We have identified in the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) an attractive and tractable target for development of drugs to treat breast cancer. IDO has been implicated in immunosuppression but its possible role in cancer has received little attention to date. Recent work has revealed that IDO is overexpressed commonly in human cancers. Moreover, it has been shown that IDO overexpression can contribute significantly to immune escape by tumor cells. We identified IDO through its genetic interaction with Bin1, a cancer suppression gene discovered in our laboratory. A large body of evidence argues that Bin1 acts to facilitate stress signaling and to restrain malignant development (DuHadaway et al., 2003a; DuHadaway et al., 2003b; Elliott et al., 2000; Elliott et al., 1999; Galderisi et al., 1999; Ge et al., 1999; Ge et al., 2000a; Ge et al., 2000b; Sakamuro et al., 1996). Loss of Bin1 expression occurs frequently in breast tumors (Ge et al., 2000a; Sakamuro et al., 1996). Our studies in a knockout mouse model have revealed that Bin1 loss leads IDO-mediated immune escape of neoplastically transformed cells. Since Bin1 loss elevates IDO, which suppresses immunity, we hypothesized that chemical inhibitors of the IDO would relieve breast tumor immunosuppression and elicit breast tumor cell death.

Body

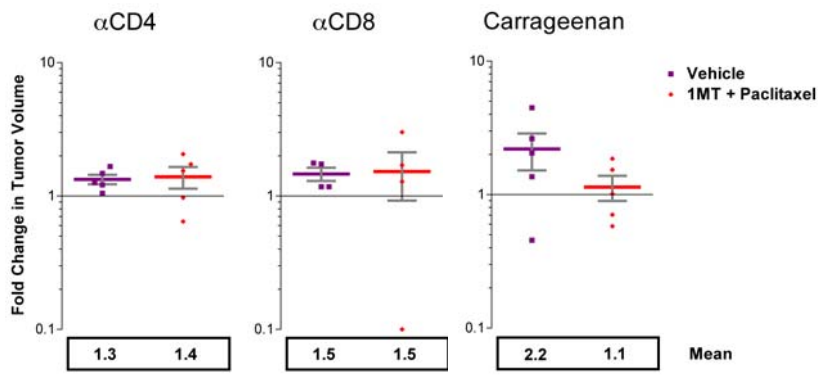
During the past year work on IDO in cancer and disease generally has exploded in the field. In our original proposal to the DoD Breast Cancer Program we proposed three aims, all of which were completed last year (as reported in the previous progress report). A large body of new work beyond the original aims continues to be done to drive clinical translation. Last year, we reported a major publication of our findings on IDO regulation and targeting for cancer treatment in *Nature Medicine* (Muller et al., 2005)*;* the successful application for NIH R01 funding of a project extending beyond the DoD funded work; the sale of a start-up company we created around our IDO therapeutic technology to a larger biopharmaceutical company, New Link Genetics Corporation (NLGC), who is driving clinical development of IDO inhibitors (to start late this year); and the characterization of several natural products including the plant phytoalexin brassinin which act as IDO inhibitors. This latter work was published recently with our chemist collaborators at Bryn Mawr College in the *Journal of Medicinal Chemistry* (Gaspari et al., 2006). Two additional publications this year from our group in the IDO area were reviews in *Expert Opinion in Therapeutic Targets* and *Cancer Research.*

The two major lines of work initiated last year were to validate and extend the 'ImmunoChemo' therapeutic principle using the IDO inhibitor 1MT in the MMTV-neu and 4T1 mouse models of breast cancer, respectively. In particular, we wished to explore pharmacological and immune dependency of the therapy in MMTV-neu mice and to examine and optimize the efficacy, short-term response, and long-term survival of the therapy in the 4T1 model (which is fully penetrant and aggressive in terms of metastatic potential, unlike MMTV-neu). We report progress in this work below. An unexpected finding also led us to clone an IDO-related gene termed INDOL1 or IDO2 for characterization. This was a big find in that the IDO field has assumed for many years that IDO is not part of a larger gene family, which our discovery shows is incorrect.

In the MMTV-neu mice, we performed immune depletion experiments by injection of monoclonal antibodies, as described previously for ectopic skin tumor experiments

(Muller et al., 2005), showing that regression of autochthonous breast tumors arising spontaneously in the transgenic model were dependent on both CD4+ and CD8+ T cells, as predicted (Fig. 1). During pharmacological studies of 1MT exposure in the mouse, which were initially performed using a subcutaneous time-release pellet formulation, we learned that although the time-release formulation was designed by the vendor to last for the entire 2 week period of the experiment, the pellets actually lost their ability to deliver detectable levels of 1MT after only 5-7 days (data not shown). For this reason, we inferred that the antitumor properties of this 1MT formulation measured under the conditions of the combination treatment at the 2 week endpoint (Muller et al., 2005) reflected a stable feature of IDO inhibition on tumor cell persistence. To test this directly, we compared the tumor response at 2 weeks after various short-term periods of combination treatment in which 1MT was delivered by oral dosing on a qd (once-a-day) or bid (twice-a-day) schedule (without change to the 3x weekly i.v. paclitaxel dosing at 13.3 mg/kg (Muller et al., 2005)). As expected, we found that 5 days of 400 mg/kg 1MT delivered on a bid schedule was sufficient to produce marked tumor regressions, with complete regressions seen in animals treated either 4 or 5 days with 1MT in this trial (Fig. 2).

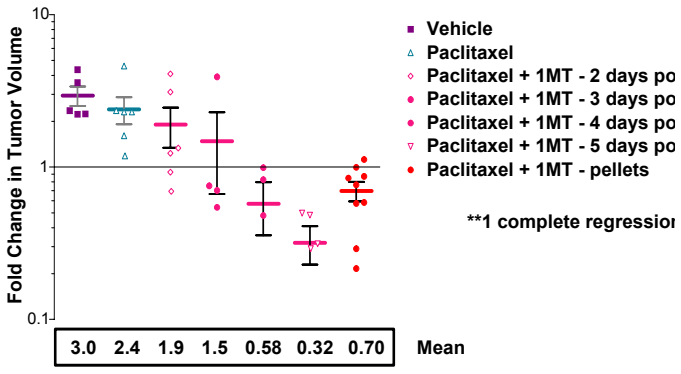
Fig. 1. Evidence that CD4+ and CD8+ T cells as well as crosspresentation are needed for regression of established MMTV-neu tumors. Tumor-bearing mice were enrolled in a 2 wk therapeutic response assay, treated with CD4 or CD8 monoclonal antibodies or carrageenan (which depletes monocyte/macrophages), an d then treated with vehicle control or combination therapies as described previously in Muller et al. (Muller et al., 2005) The horizontal line represents tumor stasis at the 2 week endpoint.



In further work, we developed and validated the antitumor properties of 1MT in the metastatic breast cancer model 4T1. In particular, we employed bioluminescent imaging to investigate short-term responses to IDO combination therapy, as well as survival

studies to investigate long-term responses to this regimen (Fig. 3,4). 4T1 is a highly aggressive and metastatic tumor cell line. We chose 4T1 for study because its metastatic prowess is manifested very quickly after injection into mice, thereby allowing an initial assessment of how well IDO inhibition might control metastatic spread (which is ultimately the cause of lethality in this model). Briefly, what we found was that the combination of 1MT plus cyclophosphamide (the most potent drug in this model) produced short-term responses that were sufficiently robust to convert into long-term survival benefits. As before, 1MT had little effect on its own and, in our hands, cyclophosphamide delivered at weekly doses of 25-100 ug/kg had only limited effects on tumor growth. In contrast, the combination of the two agents (with 1MT given daily at 400 mg/kg bid for 5 days, then 2 days off per week) produced both robust short-term responses plus a significant increase in survival. These regimens are encouraging because while they have not been optimized they are clearly active. Parallel lines of experimentation in our laboratory and in David Munn's laboratory at Medical College of Georgia using the B16 mouse melanoma cell line have produced similar results (data not shown and D.M., personal communication). Together these data are part of a manuscript currently being prepared for submission with Dr. Munn's group (D. Hou, A.J. Muller, M. Sharma, J. DuHadaway, E. Sutanto-Ward, A.L. Mellor, G.C. Prendergast, and D.H. Munn. Plasmacytoid dendritic cells distinguish stereoisomeric inhibitors of indoleamine 2,3-dioxygenase).

Fig. 2. Short-term oral dosing of 1MT in the combination regimen is sufficient to manifest robust long-term effects in the MMTV-neu breast tumor model. Tumor-bearing mice were enrolled in a 2 week combination therapy assay with 1MT and paclitaxel as described (Muller et al., 2005), except that 1MT was delivered p.o. at 400 mg/kg on a daily bid schedule for the days indicated. The s.c. time-release formulation of 1MT in the combination therapy is provided for comparison.



In other work, we continued to analyze the activity of chemical derivatives synthesized in four structural series of IDO inhibitors that we had discovered by screening

commercially available compounds containing indoleamine or known indoleamine mimetics (indoleamine thiohydantoins, brassinins, ß-carbinols, and naphthoic acids). Last year, this line of work outside of our original DoD proposal was picked up for funding by the NIH. Briefly, this year we identified several novel drug-like inhibitors of IDO in the brassinin and naphthalene series (data not shown). We essentially completed work in the thiohydantoin and brassinin series of inhibitors, where systemic toxicities (thiohydantoins) or the ability to attain high potencies (brassinins) was a concern. To date, the naphthquinone series where nanomolar levels of potency have been achieved (3 compounds of Ki=20-70 nM) seems to show the best promise.

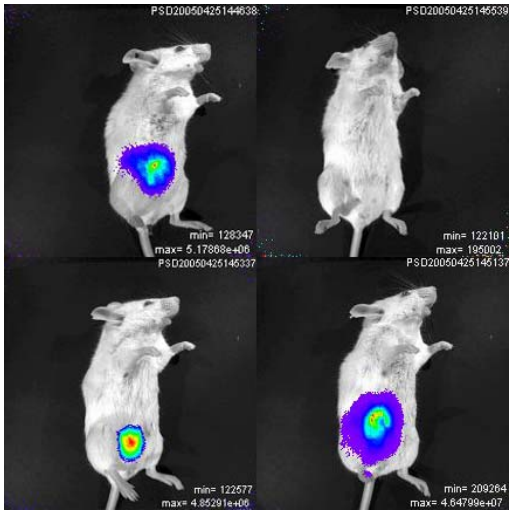


Fig. 3. Short-term response. 10e5 4T1 cells experssing a luciferase transgene were injected orthotopically into the mammary fat pads of BALB/c mice. Therapy was initiated the following day with placebo (top left), 100 mg/kg cytoxan i.p. weekly (bottom left), 400 mg/kg DL-1MT p.o. bid (bottom right), or both treatments (top right). Bioluminescence imaging was performed 7d after therapy was initiated.

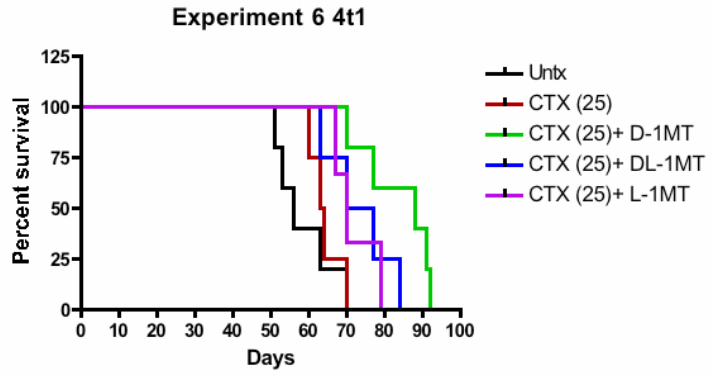
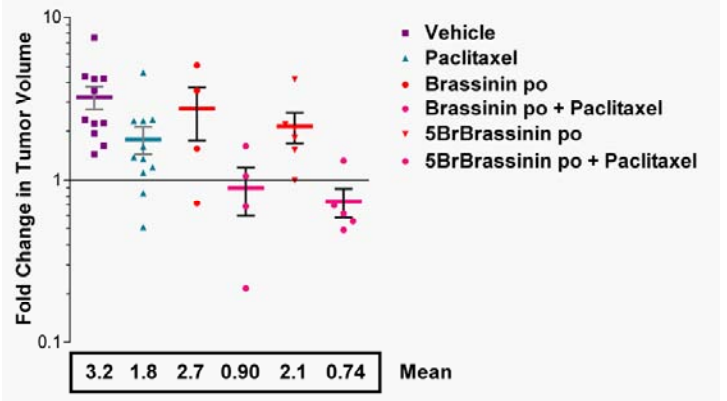


Fig. 4. Long-term response. 10e5 4T1 cells were injected orthotopically into the mammary fat pads of BALB/c mice. Therapy was initiated the following day with placebo (top left), 100 mg/kg cytoxan i.p. weekly (bottom left), 400 mg/kg 1MT p.o. bid with weekends off (bottom right), or both treatments (top right). Mice were euthanized when breast tumors exceeded >10% body weight. The D isomer of 1MT was most active in the assay, extending survival a mean of 27 d.

Our medicinal chemistry effort in the brassinin series was published this year (Gaspari et al., 2006). Brassinins are part of a natural class of compounds called phytoalexins, found in cruciferous plants such as Chinese cabbage, which are known to prevent breast cancer in animals (Mehta et al., 1995). In vivo experiments we performed in tumor-bearing MMTV-neu mice established that, consistent with their ability to inhibit

IDO in vitro and in cells (Gaspari et al., 2006), brassinins can cooperate with chemotherapy like 1MT or other IDO inhibitors identified in our laboratory (Fig. 5). These experiments indicate that as a “neutraceutical” brassinin may have utility in the treatment as well as prevention of breast cancer, as has been suggested for other cancer chemopreventative agents recently (Sarkar and Li, 2006).

Fig. 5. Novel IDO inhibitors in the brassinin series have antitumor properties in combination with chemotherapy. Tumor-bearing MMTV-neu mice were enrolled in a 2 wk trial and treated as described previously (Muller et al., 2003), except that brassinins were used as the IDO inhibitor dosed p.o. at 400 mg/kg for 5 days.



In another line of work, an unexpected set of observations prompted us to initiate a search for IDO isoforms that ultimately led us to discover a novel IDO-related gene called INDOL1/IDO2. The stimulus to this line of work was the following. In all our experiments until recently, we had been using a racemic mixture of the IDO inhibitor D,L-1MT. A comparison of the stereoisomers led to the surprising observation that D-1MT was somewhat more active as an antitumor compound but that it did not inhibit the recombinant IDO enzyme we and others in the field have used. Similar results from the Munn laboratory were extended by the finding that D-1MT could nevertheless inhibit tryptophan catabolism in dendritic cells, but not other cells where IDO was known to be expressed. Together, these observations implied that (a) alternately processed or modified isoforms of IDO might exist in dendritic cells that could be inhibited by D-1MT; (b) D-1MT was isomerized to L-1MT in dendritic cells and perhaps other cells in vivo; or (c) that D-1MT acted upon a tryptophan catabolizing enzyme other than IDO that was expressed selectively in dendritic cells.

Our efforts to consider these different solutions to the problem posed by the biological activity of D-1MT led us to look at IDO splicing patterns and IDO-related sequences

generally in the mouse and human genomes. Historically, it was thought that IDO is unique and not part of a gene family. However, through careful database searches we identifed related sequences that were inaccurately annotated in the human genome but not the mouse genome as a distinct and related gene. The primary amino acid sequence of the gene we have termed IDO2 is ~44% conserved to IDO (Fig. 6). (In the mouse genome a very recent change in the annotation in late April 2006 changed the nomenclature from the anonymous reference LOC209176 to INDOL1, for INDO-like-1.) IDO and IDO2 are somewhat more distant that most gene family members and IDO2 is better conserved mouse-to-human than the corresponding IDO genes. Notably, despite their relative sequence divergence, IDO2 conserves all the critical animo acids known to be important for catalytic function in IDO {Sugimoto, 2006 #2499), suggesting that IDO2 a similar substrate specificity and catalytic mechanism to IDO.

Fig. 6. Similarity of human IDO2/INDOL1 to human IDO. BLASTP comparison of IDO (query) to IDO2/INDOL1 (sbjct).

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 Score =  328 bits (841),  Expect = 3e-88
 Identities = 172/390 (44%), Positives = 248/390 (63%), Gaps = 5/390 (1%)

Query  30   ESYHISEEYGFLLPDSLKELPDHYRPWMEIANKLPQLIDAHQLQAHVDKMPLLSCQFLKG  89
            + YHI EE GF LP+  + LPD Y  WM IA  LP LI++ QL+  V+K+ +LS   L
Sbjct  13   KEYHIDEEVGFALPNPQENLPDFYNDWMFIAKHLPDLIESGQLRERVEKLNMLSIDHLTD  72

Query  90   HREQRLAHLVLSFLTMGYVWQEGEAQPAEVLPRNLALPFVEVSRNLGLPPILVHSDLVLT  149
            H+ QRLA LVL  +TM YVW +G     +VLPRN+A+P+ ++S+ L LPPILV++D VL
Sbjct  73   HKSQRLARLVLGCITMAYVWGKGHGDVRKVLPRNIAVPYCQLSKKLELPPILVYADCVLA  132

Query  150  NWTKKDPDGFLEIGNLETIISFPGGESLHGFILVTALVEKEAVPGIKALVQATNAILQPN  209
            NW KKDP+  L   N++ + SF  G+   GF LV+ LVE  A   IK +     A+
Sbjct  133  NWKKKDPNKPLTYENMDVLFSFRDGDCSKGFFLVSLLVEIAAASAIKVIPTVFKAMQMQE  192

Query  210  QEALLQALQRLRLSIQDITKTLGQMHDYVDPDIFYAGIRIFLSGWKDNPAMPAGLMYEGV  269
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Sbjct  313  NPSVREFVLSKGDAGLREAYDACVKALVSLRSYHLQIVTKYILIPA--SQQPKENKTSED  370

Query  387  PQALKDRGTGGTAVMSFLKSVRDKTLESIL  416
            P  L+ +GTGGT +M+FLK+VR  T +S+L
Sbjct  371  PSKLEAKGTGGTDLMNFLKTVRSTTEKSLL  400
```

Interestingly, IDO2 appears to have a more restricted pattern of expression than IDO, being most highly expressed in dendritic cells treated with the immune suppressive cytokine IL-10 (http://cgap.nci.nih.gov.proxy1.lib.tju.edu:2048/SAGE/FreqsOfTag?ORG=Mm&METHOD=SS10,LS10&FORMAT=html&TAG=CTGCTGCTGC). Strikingly, in the human and mouse genomes the IDO2 gene is located on chromosome 8p12 immediately downstream of the IDO gene, suggesting that this gene family may have emerged relatively recently in evolution by gene duplication. While we do not as yet have full information about the enzymology of IDO2, its germane significance derives from the possibility that it might be a target for D-1MT, implicating IDO2 in antitumor responses in the manner of IDO. We are currently engaged in experiments to test the ability of IDO2 to be inhibited by all the other small molecules we initially defined as IDO inhibitors.

In addition to these activities, this year we worked with CTEP (which picked up the IDO project from RAID based on the increasing interest of 1MT as a realistic proof-of-concept molecule, stimulated to significant extent by our *Nature Medicine* paper) and with New Link Genetics Corporation (who has licensed IDO technology from our group at LIMR and the Munn/Mellor group at MCG) to obtain results for an IND application for Phase 0/1 trials to be conducted at NCI and Fox Chase Cancer Center, the latter near our laboratories in Philadelphia. Collaborations initiated with Drs. David Munn and Andrew Mellor, leaders in the field of IDO and immune regulation, are focused on the 1MT mechanism of action and the role of IDO in carcinogenesis, respectively. With Dr. Munn, we have prepared a manuscript for submission that addresses the mechanism of action of D-1MT, the lead clinical candidate for proof of concept trials in humans. With Dr. Mellor, we are employing his IDO knockout mouse model to perform carcinogenesis and therapeutic response experiments aimed at genetically validating the role of IDO as an oncogene in breast cancer as well as the role of IDO as the target of 1MT and other small molecule inhibitors of this enzyme.

Last year, we had proposed the following work. First, we had proposed to crystallize the IDO enzyme with an inhibitor, to promote structural studies to aid drug design. However,

we ceased this line of work when it was reported by a Japanese group earlier this year {Sugimoto, 2006 #2499}. The findings of this group are being employed by our chemist collaborators to refine our lead inhibitors, particularly in the naphthalene series. Second, we had also proposed to refine the pharmacodynamic assay for assessing in vivo potency. This is one area where we have not made good progress, the kynurenine detection in mouse serum remaining a problematic issue in terms of reproducibility. We are currently reaching out to the CTEP group at NCI to address this deficiency in our assay prowess in this area. Lastly, we also had proposed to complete investigations of formulating IDO inhibitors including brassinins in ß-cyclodextrin. On this question, we have determined concentrations which reliably and reproducibly enhance the exposure of brassinin compound (data not shown). The latest in vivo results on brassinin and a related bioactive compound 5-bromo-brassinin are presently in preparation for submission. Finally, we continue to investigate what immune cells are critical in the anticancer response to IDO inhibitors, for example, confirming that CD4+ and CD8+ T cells are critical and that IDO inhibition can be partially phenocopied by CTLA-4-Ig, which depletes CD25+ T regulatory cells. This finding extends other evidence suggesting that depleting CD25+ T cells in cancer can improve therapeutic responses. In summary, the DoD funded work on IDO has gone far beyond the initial aims to generate a innovative and exciting translational project for breast cancer treatment.

Key Research Accomplishments – Year 3

1. Synthesis and evaluation of the IDO inhibitory properties of brassinins (Gaspari, P., Banerjee, T., Malachowski, W.P. Muller, A.J., Prendergast, G.C., DuHadaway, J., Bennett, S. and Donovan, A.M. (2006). Structure-activity study of brassinin derivatives as indoleamine 2,3-dioxygenase inhibitors. J. Med. Chem., **49,** 684-692). This work establishes a likely mechanism of action for brassinins in IDO inhibition. In vivo experiments suggest a use for brassinins in the treatment as well as prevention of breast cancer through the IDO mechanism (Fig. 5 above).
2. Confirmation of the expectation that CD4+ and CD8+ T cells are required for IDO inhibitors to elicit regression in combination with chemotherapy in the MMTV-neu transgenic mouse model of breast cancer (Fig. 1 above).
3. Demonstration that short-term oral administration of 1MT in tumor-bearing MMTV-neu mice is sufficient to elicit stable antitumor responses (Fig. 2 above). This data will be included in the collaborative manuscript to be submitted with Dr. Munn's group (Hou et al. Plasmacytoid dendritic cells distinguish stereoisomeric inhibitors of indoleamine 2,3-dioxygenase).
4. Demonstration that in combination with cyclophosphamide 1MT can elicit robust short-term and long-term antitumor responses in the aggressive and highly metastatic 4T1 breast cancer model system (Figs. 3,4 above). This data will be included in the collaborative manuscript to be submitted with Dr. Munn's group (Hou et al. Plasmacytoid dendritic cells distinguish stereoisomeric inhibitors of indoleamine 2,3-dioxygenase).
5. Cloning of a novel IDO-related gene termed IDO2/INDOL1 (Fig. 6 above).

Reportable Outcomes

1. Brassinins inhibit IDO and preclinical experiments show that they can be dosed in combination with chemotherapy to improve antitumor efficacy in breast cancer.
2. Short-term oral dosing of an IDO inhibitor can elicit robust short-term and long-term antitumor responses in preclinical animal models of breast cancer.
3. High potency small molecule inhibitors of IDO were identifed in the naphthoic acid structural series (potencies of 10-100 nM).
4. A new IDO-related gene termed IDO2 has been discovered which may itself be a target for immune regulation by small molecule inhibitors of IDO.
5. The unusually rapid translation of IDO inhibitors from an obscure stage of preclinical study to Phase I clinical trials seeded by this DoD project represents an unusual and notable success for the DoD BRCA Program.

Conclusions

Over the past year, interest in IDO as a modulator of T cell immunity including in cancer has exploded. IDO is involved in multiple disease states and other enzymes in the tryptophan catabolism pathway regulated by IDO are being linked to disease (e.g. (Giorgini et al., 2005; Platten et al., 2005; Wolf et al., 2004). The rapidly growing biomedical literature on the importance of this enzyme in cancer and other diseases is exciting. One of the chief successes of this project is the collaborative effort it helped seed between the NCI, a private biotech company (New Link Genetics), and two academic research organization (LIMR and MCG) who are working now to move a lead IDO inhibitory compound into Phase I clinical trials later this year. Much of the excitement generated around IDO inhibitors for cancer would not have been possible without the work on this project, which when published last year in *Nature Medicine* put IDO firmly on the map of targets for cancer treatment.

The original aims of the the proposal – to gain support for the general hypothesis that IDO inhibitors offer a novel modality to enhance breast cancer therapy – are complete. Aim 1 to generate an IDO monoclonal antibody was successful. This reagent has been

commercialized to make it readily available to the field and most of the many publications over the past year reporting the use of an IDO antibody have used this reagent, illustrating its value in this rapidly developing field. Aim 2 to develop IDO inhibitors was successful and expanded dramatically into two NIH supported project (R01 and RAND awards). Aim 3 to evaluate the antitumor properties of an IDO inhibitor was successful and expanded dramatically in two breast cancer model systems. A significant multidisplinary collaboration was built around the success of Aims 2 and 3, involving the NCI, a biotechnology company, and three academic research organizations (LIMR, Bryn Mawr College, and Medical College of Georgia) to aggressively translate preclinical findings into organization of a Phase I clinical trial to be conducted later this year. Additional achievements of this project were the identification and characterization of brassinins, natural compounds that inhibit IDO and tumor survival; development of oral formulations of IDO inhibitors; and cloning of a novel IDO-related gene which may be relevant to the antitumor properties of IDO inhibitors. By any measure, the achievements of this project present a laudable success for the DoD BRCA Program.

Project Reports, Reviews, and Manuscripts (chronological order)

Muller, A.J., DuHadaway, J.B., Donover, P.S., Sutanto-Ward, E., and Prendergast, G.C. (2005). Inhibition of indoleamine 2,3-dioxygenase, a target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nature Med. 11, 312-319.

Muller, A.J., Malachowski, W.B., and Prendergast, G.C. (2005). IDO in cancer: targeting pathological immune tolerance with small molecule inhibitors. Expert Opin. Ther. Targets 9, 831-849.

Muller, A.J. and Prendergast, G.C. (2005). Marrying immunotherapy with chemotherapy: why say IDO? Cancer Res. 65, 8065-8068.

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The following personnel received pay from this research effort:

Donover, Scott

DuHadaway, James

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Ramalingham, Arivadanambi

Appendix

Reprints provided:

Muller, A.J., Malachowski, W.B., and Prendergast, G.C. (2005). IDO in cancer: targeting pathological immune tolerance with small molecule inhibitors. Expert Opin. Ther. Targets 9, 831-849.

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Indoleamine 2,3-dioxygenase in cancer: targeting pathological immune tolerance with small-molecule inhibitors

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Indoleamine 2,3-dioxygenase (IDO) is an interferon (IFN)-γ-inducible, extrahepatic enzyme that catalyses the initial and rate-limiting step in the degradation of the essential amino acid tryptophan. Elevated tryptophan catabolism mediated by IDO is associated with a wide variety of human cancers and has historically been thought to be a tumoricidal consequence of IFN-γ exposure. Evidence of a physiological requirement for IDO activity in protecting the allogeneic fetus from rejection by the maternal immune system has stimulated a radical shift in thinking about the role of IDO in cancer. Evidence now suggests that tumours can exploit IDO-mediated peripheral tolerance to promote immune escape. This review summarises key studies that implicate IDO as an important mediator of peripheral immune tolerance as well as the development of a promising new anticancer modality that incorporates the use of IDO inhibitors. The second part focuses on the current state of development of IDO inhibitory compounds as potential pharmaceutical agents.

Keywords: 1-methyl-tryptophan (1MT), antigen-presenting cell (APC), Bin1, chemotherapy, dendritic cell (DC), *Indo*, indoleamine 2,3-dioxygenanse (IDO), macrophage, tryptophan, tumour

Expert Opin. Ther. Targets (2005) 9(4):831-849

1. Introduction

Tumour interactions with the host immune system are complex and dynamic. Inflammation produces a tumour-promoting environment comprised of cytokines, chemokines and growth factors, activated stroma, and DNA damaging agents [1]. However, tumours are also subject to immune surveillance; in particular, the expression of tumour antigens means that cancer cells must evolve mechanisms to escape or subvert antitumour immunity in order to successfully progress [2,3]. This process of 'immune editing', whereby immune-mediated destruction of nascent cancer cells provides selective pressure that shapes the immunogenic phenotype of the growing tumour [4], has been clearly demonstrated in mouse tumour models [5]. Studies of human tumours provides further evidence of a microenvironment of immune privilege that protects cancer cells from immune destruction [6-8]. Now widely recognised as an additional 'hallmark of cancer' [9], immune escape is proving to be an important obstacle to the development of immunotherapeutic protocols such as adoptive immunotherapy of *in vitro* activated T cells, which has been only marginally successful despite evidence that the transferred T cells can localise to tumours [10,11]. A promising target for attacking tumoural immune escape, reviewed here, is the enzyme indoleamine 2,3-dioxygenase (IDO). In particular, recent data show that IDO inhibitors can cooperate with cytotoxic agents to more effectively destroy tumours, in line with the burgeoning notion that combining immunotherapeutic and chemotherapeutic treatment modalities can be remarkably effective.

2. Indoleamine 2,3-dioxygenase, immune regulation and cancer

2.1 Indoleamine 2,3-dioxygenase: background

Elevated tryptophan catabolism, a condition previously associated with microbial infections such as tuberculosis, was observed in patients with bladder cancer in the 1950s [12]. By the 1960s, elevated levels of tryptophan catabolites had been documented in the urine of patients with a variety of malignancies including leukaemia, Hodgkin's disease, prostate disorders, and breast cancer [13-18]. The hepatic enzyme tryptophan dioxygenase (TDO2; EC 1.13.11.11) was known at the time to carry out the catabolism of dietary tryptophan, having been the first inducible mammalian enzyme to be isolated back in the 1930s [19,20]. TDO2 catalyses the initial and rate-limiting step in the degradation of tryptophan to *N*-formylkynurenine. However, no increase in TDO2 activity was detected in patients presenting with elevated tryptophan catabolites, implying the activity of a second enzyme.

In 1963 the isolation of a non-hepatic tryptophan catabolising enzyme, D-tryptophan pyrrolase, was first reported [21,22]. Renamed indoleamine 2,3-dioxygenase (IDO; EC 1.13.11.17), this enzyme also converts tryptophan to *N*-formylkynurenine. However, despite producing the same reaction product as TDO2, IDO is otherwise remarkably dissimilar [23]. IDO is a monomeric 41 kDa enzyme whereas the active TDO2 enzyme is a tetramer totaling 320 kDa in size. The two proteins are antigenically distinct [24] and share no significant amino acid sequence homology (as determined by standard comparative analysis using the NCBI 'BLAST 2 Sequences' online program). IDO has less stringent substrate specificity, cleaving a number of indole-containing compounds that are not recognised by TDO2. This is an advantageous consideration in the development of compounds that will selectively inhibit IDO but not TDO2, because the IDO active site is likely to accommodate a wider spectrum of inhibitory compounds than TDO2. IDO is a haem-containing enzyme that utilises superoxide anion for activity, whereas TDO2 does not use superoxide as an oxygen donor. *In vitro*, IDO enzyme reactions are performed by substituting ascorbic acid for superoxide. The IDO enzyme also requires methylene blue as a cofactor in the reaction to maintain full activity. *In vivo*, the role of methylene blue is thought to be assumed by either a flavin or tetrahydrobiopterin. The cofactor binding site is distinct from the substrate binding site [25] and may represent an opportunity for the development of distinct classes of noncompetitive IDO inhibitors.

Indo is the official designation for the gene encoding the IDO enzyme. In humans, *Indo* is a single copy gene comprised of 10 exons spanning ~ 15 kb which maps to 8p12-p11 [26,27]. The mouse gene, also located on chromosome 8, has a similar genomic organisation. There is, however, a good deal of divergence at the primary amino acid sequence level between species, with the human and mouse *Indo* genes sharing only 62.5% identity. The *Indo* gene is found early in evolution with a homologous gene present in the yeast *Saccharomyces cerevisea* (Genbank no. – Z49578). It appears, however, to have undergone functional divergence during the course of the evolution of archaegastropod mollusks (including *Sulculus*, *Nordotis*, *Battilus*, *Omphalius* and *Chlorostoma*), which express a unique form of myoglobin derived from the primordial *Indo* gene [28]. This abalone myoglobin provides useful structure/function data regarding the mammalian IDO enzyme. In particular, a mutation of a conserved histidine, which was determined to be the most likely iron-bound proximal histidine for the abalone myoglobin, has been shown to also be critical for mammalian IDO activity ([29] and PS Donover, J DuHadaway, AJ Muller, GC Prendergast, unpublished results). Solving the crystal structure of human IDO (for which diffraction data at 2.3 angstrom resolution has been collected according to a recent web posting (S Oda, H Sugimoto, T Yoshida, Y Shiro, unpublished results) will be immensely valuable for structure–activity relationship-based modelling of inhibitory compound interactions.

The cytokine IFN-γ is a major inducer of IDO, especially in antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs) [30-33]. Transcriptional induction of the *Indo* gene is mediated through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway; in particular, JAK1 and STAT1α [34]. STAT1α appears to act to induce *Indo* gene expression both directly through binding of IFN-γ activation sites (GAS) within the *Indo* promoter as well as indirectly through induction of interferon regulatory factor (IRF)-1 which binds the *Indo* promoter at two IFN-stimulated response element sites (ISRE) [34-38]. NF-κB also contributes to *Indo* induction [34]. In particular, IFN-γ and TNF (which signals through NF-κB) appear to act synergistically to induce expression of IRF-1 through a novel composite binding element for both STAT1α and NF-κB in the IRF-1 promoter (termed a GAS/κB element) that combines a GAS element overlapped by a non-consensus site for NF-κB [39]. A possible alternative to the development of inhibitors that block IDO enzyme activity directly might be to develop compounds that block the induction of *Indo* gene expression. Given the number of other targets regulated by IFN-γ signalling, however, it is likely that blocking the entire signalling pathway would have pleiotropic consequences unrelated to IDO inhibition.

2.2 IDO promotes peripheral immune tolerance

The topic of the role of IDO in peripheral tolerance has already been extensively covered in a number of recent reviews including [40-42], and so is not treated exhaustively here. IDO activity is ubiquitously present, albeit at differing levels, in mammalian organs [43,44]. Like TDO2, IDO catalyses the initial and rate limiting step in the *de novo* biosynthesis of the critical coenzyme nicotinamide adenine dinucleotide (NAD). Unlike TDO2, however, IDO is unresponsive to changes in tryptophan or glucocorticoid levels [43] and is, therefore, unlikely to be responsible for metabolic processing of dietary tryptophan uptake. *N*-formylkynurenine generated by IDO is rapidly converted by a ubiquitously expressed formamidase to kynurenine,

but most mammalian tissues outside of liver lack the subsequent downstream enzymes necessary for *de novo* NAD biosynthesis. Instead, the kynurenine generated by IDO predominantly enters the bloodstream for urinary elimination [44] although some other cell types, including certain immune and neuronal cells, can further metabolise tryptophan to quinolate and even NAD [45]. Particularly high basal levels of IDO activity have been observed within the epididymis, the placenta of pregnant females and at other sites of immune privilege [23,46-49]. Bacterial lipopolysaccharide (LPS) exposure elevates IDO activity in a variety of mouse tissues, most notably colon and lung [43,44,50]. The pattern of IDO expression suggested early on a possible role in inflammation [51], and accumulating experimental evidence supported the hypothesis that IDO might protect the host from auxotropic pathogens by depleting the local tryptophan pool and/or the production of toxic catabolites [52,53]. After IDO activity was found to be elevated in various cancer patients, the hypothesis was expanded to include the idea of IDO upregulation being a tumoricidal consequence of IFN-γ exposure through both starvation of the proliferating tumour cells of the essential amino acid tryptophan as well as exposure to cytotoxic tryptophan catabolites [54-58].

In 1998 Munn, Mellor and co-workers published the seminal finding that IDO activity appears to be essential for protecting the allogeneic fetus from the maternal immune system [59]. This study was performed based, in part, on *in vitro* findings indicating that T cells are exquisitely sensitive to tryptophan depletion by macrophages. Upon encountering an activation signal in a low tryptophan environment, T cells were found to be unable to complete progression through the cell cycle, arresting in mid-G1. If not activated again in the presence of sufficient tryptophan, these T cells subsequently underwent apoptosis [60]. The demonstration that macrophages are imbued with the ability to suppress T-cell proliferation when differentiated *in vitro* through exposure to macrophage colony-stimulating factor (MCSF) [61,62] had indicated that this might be a useful experimental system to study the mechanistic basis for the establishment of peripheral tolerance. Tryptophan depletion mediated by increased IDO activity was shown to be upregulated in MCSF-differentiated macrophages responding to IFN-γ stimulation [60]. In this *in vitro* study, the small-molecule IDO inhibitor 1-methyl-DL-tryptophan (1MT) was used to block macrophage IDO activity. 1MT had been identified as the most effective inhibitor among a small series of tryptophan analogues evaluated for IDO inhibitory activity [63]. A detailed review of the literature on small-molecule inhibitors of IDO can be found in Section 3.

1MT became the key tool employed *in vivo* to demonstrate the essential role of IDO in protecting the developing fetus from maternal immunity [60]. 1MT was delivered by subcutaneous implantation of time-release pellets, which permitted continuous dosing to be achieved. An important caveat with regard to this study, as well as all subsequent studies employing 1MT that have been published, to date, is that none has actually demonstrated that direct inhibition of the intended target, IDO, is responsible for the observed biological consequences. For instance, it has been reported that the transport system L [64,65], which transports L-tryptophan into cells, can also be inhibited by 1MT. This could contribute to the apparent biological activity of 1MT, independent of any direct inhibitory effect on IDO, by potentially limiting access of IDO to substrate and perhaps even directly blocking access of T cells to tryptophan. It has not yet even been demonstrated that IDO is inhibited by 1MT *in vivo* at the dose levels delivered nor has there been any reported attempt to correlate biological effects of 1MT with IDO pharmacodynamics. Therefore, the possibility that 1MT may be acting through an off-target mechanism of action has yet to be rigorously addressed.

In another ground-breaking finding, IDO has been implicated in the suppression of T-cell activation by cytotoxic T lymphocyte antigen (CTLA)-4 [66]. CTLA-4 is an important mediator of peripheral immune tolerance, and mice that are genetically deficient for CTLA-4 develop fatal autoimmune disease [67,68]. CTLA-4 belongs to the CD28 family of proteins. CD28 is an important co-stimulatory molecule for activation of T cells through engagement of the T-cell receptor (TCR). Both CD28 and CTLA-4, which are expressed on the surface of T cells, bind the B7 ligands, B7-1 and B7-2, expressed on the surface of APCs. The soluble fusion protein CTLA-4-immunoglobulin (CTLA4-Ig), which also binds B7-1 and B7-2, can prevent allograft and xenograft rejection in mouse transplantation models [69-71]. It has generally been accepted that CTLA-4 is directly antagonistic to CD28 in T cells, either through out-competing CD28 for access to B7 ligand, inducing immunosuppressive cytokines, or directly interfering with CD28-mediated and/or TCR-mediated signalling [72]. The first evidence that IDO might be an important mediator of CTLA-4-Ig-induced tolerance was the observation that, in a diabetic mouse model, the ability of CTLA-4-Ig to effectively suppress immune rejection of pancreatic islet allografts was lost if IDO activity was concurrently inhibited by treatment with 1MT [66]. The study by Grohmann *et al.* further suggested that CTLA-4-Ig-mediated tolerance occurs through a heterodox mechanism of 'reverse' signalling through B7 molecules on APCs, which promotes IFN-γ production to induce IDO. Subsequent studies have provided further support for and refinement of this model [73-78], which is consistent with CTLA-4 expression at the maternal–fetal interface during gestation [79].

2.3 IDO in tumoural immune escape

The concept that IDO activity is physiologically important for establishing peripheral tolerance to alloantigens expressed by the fetus has engendered a complete rethinking of the implications of the elevated IDO activity observed in cancer patients. Induction of IDO was generally thought to be a turmoricidal consequence of IFN-γ exposure as the growing tumour cells were starved of an essential amino acid as well as exposed to

toxic products of tryptophan degradation [55,80-82]. However, cancer cells are highly adaptive, compensating for a low tryptophan environment, for instance, by upregulating tryptophan tRNA synthetase induction in response to IFN-γ [83]. If IDO can block immune responses to the highly antigenic paternally-derived alloantigens expressed by the fetus, it should also be capable of blocking responses to much weaker tumour antigens. Therefore, tumours that can survive the deleterious consequences of IDO upregulation may benefit from its immune suppressive activity. The idea that IFN-γ exposure can have diametrically opposed consequences for tumours is already well-established in the literature. For instance, IFN-γ has been shown to cooperate with lymphocytes to protect against the development of both spontaneous as well as chemically-induced tumours, but the tumours that do grow out in this context are more aggressive when transplanted into a syngeneic, immunocompetent host [5]. This is consistent with positive selection for reduced immunogenicity, a phenomenon that has been termed 'immune editing' [4].

Does the relevant upregulation of IDO activity occur in the tumour cells themselves or in the adjacent stroma? Two competing, although not necessarily mutually exclusive explanations have developed regarding this question. Experimental evidence supports the idea that cancer cells with active IDO enzyme can promote immune suppression. A fibrosarcoma cell line ectopically expressing IDO has been shown to directly inhibit T-cell responses [84]. In a separate study, ectopic expression of IDO in a mastocytoma cell line promoted tumour formation in mice that should otherwise have been rendered resistant because of preimmunisation [85]. Coupled with the reports of high IDO expression in many tumour-derived cell lines [20] as well as in a high proportion of primary tumour cells from a wide range of tissues [85], it appears likely that direct expression of IDO in tumours can contribute significantly to immune escape.

This raises the question of how IDO becomes dysregulated in tumour cells. One possible answer to this question has come from the authors' own studies of the *Bin1* cancer suppression gene. Loss or attenuation of normal Bin1 protein expression during malignant progression has been described in a variety different human tumours including breast cancer, prostate cancer, melanoma, neuroblastoma [86-89] and colon cancer (K Xie, L Wang, JD, AJ Muler, GC Prendergast unpublished results). To study the mechanistic basis for the apparent selective pressure against *Bin1*, the authors used a combination of *Myc* and *Ras* oncogenes to transform primary epithelial skin cells (keratinocytes) obtained from *Bin1*-deficient neonates as well as heterozygous control littermates. The growth characteristics of these transformed cells were virtually indistinguishable *in vitro*; however, when transplanted subcutaneously into syngeneic animals, the *Bin1*-null cells were aggressively tumorigenic whereas the *Bin1*-expressing cells were not. Immune escape was implicated as Bin1-expressinng cells showed equivalent tumorigenicity when injected into nude mice. Prompted by reports that *Bin1* could impact STAT and NF-κB signalling pathways, the authors discovered that Bin1 is involved in the regulatory control of IDO. IFN-γ-mediated induction of IDO is enhanced in *Bin1*-null keratinocytes and in *Bin1*-null macrophages as well. As anticipated, treatment with the IDO inhibitor 1MT significantly suppressed the outgrowth of *Bin1*-null MR-transformed keratinocyte tumours in syngeneic animals but had no significant impact on their outgrowth in athymic nude mice [90]. These data provide further support for the conclusion that direct expression of IDO in tumour cells is capable of promoting immune escape and indicate that loss of *Bin1* is one mechanism through which IDO dysregulation may occur.

On the other hand, IDO has been implicated in immune escape by tumours that show no direct evidence of *Indo* gene expression. It has been argued that this may even be the more relevant means of establishing tolerance [91], although the details on how this might be achieved are still sketchy. Tumours formed by Lewis lung carcinoma cell line, which did not directly express detectable IDO, induced IDO upregulation in the draining lymph nodes and treatment of mice with the IDO inhibitor 1MT delayed the outgrowth of these tumours [92]. The stromal cells most likely to be providing the IDO activity in this scenario are APCs such as DCs or macrophages. Not all APCs appear to induce IDO, however, and a number of cell surface markers that may help characterise particular APC subsets that are key to mediating this immune regulatory mechanism have been reported [73,76,93-95]. In particular, a plasmacytoid class of mouse dendritic cells, which express B cell surface markers and may originate from the B cell lineage, appear to be important expressors of IDO [94,95]. Speculative mechanisms for how tumours induce IDO in proximal APCs are suggested by experimental systems in which evidence for IDO-mediated tolerance has been demonstrated. As described previously, CTLA-4 co-receptor has been implicated in the induction of IDO in APCs through B7 ligation. CTLA-4 is highly expressed on regulatory T cells (T_{reg}) which have been implicated in mediating IDO induction in the DC population [75]. T_{reg} recruitment or generation at the tumour site might thus be a mechanism for cancer cells to indirectly promote local upregulation of IDO activity. Another co-receptor, 4-1BB, has recently been implicated in the promotion of tolerance in the collagen-induced arthritis model in mice [96]. In this case, ligation of 4-1BB with an antibody stimulates the accumulation of $CD11b^+$ $CD8^+$ cells that produce high levels of IFN-γ. This induces IDO in responsive APCs which leads to tolerisation. Interestingly, aberrant expression of 4-1BB ligand has been reported in solid tumours [97,98], suggesting the possibility that tumours that do not directly express IDO might signal through 4-1BB as an alternative mechanism to induce local IDO activity.

Divergent opinions also exist as to the mechanism by which IDO promotes immune suppression, namely whether this is due to the local depletion of tryptophan levels or the local accumulation of toxic tryptophan catabolites. Of course,these two possibilities are not necessarily mutually exclusive.

In vitro data favouring each model has been reported. Shortly after publishing the fetal protection study, Munn, Mellor and co-workers published an *in vitro* study that demonstrated that T cells are exquisitely sensitive to tryptophan levels during activation [60]. Low tryptophan levels in the media promoted cell cycle arrest and eventually apoptosis if a subsequent activating signal in the presence of sufficient tryptophan was not encountered. These and subsequent experiments indicating that metabolic products were neither necessary nor sufficient for IDO-mediated inhibition in mixed lymphocyte reactions but that the tryptophan depleting effect of IDO was required [99] formed the basis for the tryptophan depletion model. However, evidence reported from other laboratories that tryptophan catabolites are primarily responsible for suppressing T cell activation [100-102], has bolstered the counter argument that tryptophan depletion by IDO is unlikely to account for the observed biology [45]. It is apparent that *in vitro* systems are too malleable to convincingly resolve this issue and that clear and definitive *in vivo* experiments will be required.

2.4 Targeting IDO as a therapeutic strategy for cancer treatment

The idea that IDO activity might protect tumours from the host immune system suggests that IDO inhibitors might have utility as anticancer agents. Given that 1MT is known to be biologically active in defeating immunological tolerance of allogeneic concepti, it has clearly been the compound of choice to perform pilot studies. The authors' own work and that of others has shown that 1MT exhibits some efficacy as a monotherapy. In tumour growth inhibition studies (treatment initiated prior to or concurrent with tumour challenge [103]) involving tumour models that either directly or indirectly utilise IDO for immune escape, 1MT treatment did cause significant tumour growth delays, but failed to block establishment [85,92]. In a more stringent type of study in which treatment was initiated on established tumours, inhibition of tumour growth with 1MT treatment alone was also observed, however, regression of tumours, a critical preclinical criterion, was not achieved [90]. These findings suggest that IDO inhibitor-based, single-agent immunotherapy may have only limited antitumour activity. Pilot experiments performed in our laboratory combining 1MT treatment with injection of IFN-γ or IL-12 did not achieve any stronger effects than 1MT alone (AJ Muller, J DuHadaway, GC Prendergast, unpublished results). It is not particularly surprising that the response of tumours is less dramatic than that of allogeic concepti, because the tumour antigens that they express are substantially less antigenic than alloantigens and the tumours may be more flexible in employing alternative mechanisms to protect themselves from immune responses as well.

The authors have further explored the use of 1MT in combination with other agents to treat established tumours in the well-accepted MMTV-*Neu* 'oncomouse' model of breast cancer in which overexpression of the HER2/ErbB2/Neu proto-oncogene drives the formation of mammary gland adenocarcinomas that closely resemble human ductal carcinoma *in situ* [104]. In particular, the authors have investigated the antitumour effects of combining 1MT with paclitaxel and other cytotoxic chemotherapeutic drugs. Such drugs might appear to be a counterintuitive choice because they can kill the immune cells that IDO inhibitors are supposed to activate. However, reports that cytotoxic drug regimens can actually promote immune cell infiltration of tumours and antitumour responses [105-108] largely prompted this line of investigation. Paclitaxel treatment by itself produced only growth inhibition of MMTV-*Neu* tumours consistent with published evidence that Neu overexpression in breast cancer cells confers paclitaxel resistance [109]. 1MT was delivered to tumour-bearing MMTV-*Neu* mice by subcutaneous introduction of time-release pellets: the same delivery route that achieves sufficient 1MT exposure for rejection of allogenic concepti. In contrast to results obtained with 1MT monotherapy, treatment of tumour-bearing MMTV-*Neu* mice with a combination of 1MT + paclitaxel resulted in tumour regression [90].

Control experiments in which pellets were infused with D,L-tryptophan (analogous to the D,L racemic mixture of the 1MT used) did not replicate the observed cooperative effect of 1MT. Thus, the observed effect could not be trivially ascribed to a nonspecific toxicity caused by high-dose of the tryptophan-like compound. The authors did not rule out the possibility of a pharmacokinetic effect of 1MT on paclitaxel, which might increase its effective dose in the mouse. However, this explanation seems unlikely because no evidence of neuropathy (e.g., hind leg dragging) that would be produced in mice by a higher effective dose of paclitaxel was observed. Immune depletion experiments as well as grafting experiments in nude mice confirmed that T-cell-mediated immunity is essential for the combination therapy to elicit tumour regression.

Similar cooperativity was observed with other chemotherapeutic agents tested, but was not universal. The DNA damaging agents cisplatin, cyclophosphamide and doxorubicin exhibited cooperativity, but the antimetabolites 5-fluorouracil and methotrexate did not. Interestingly, the other mitotic inhibitor tested, vinblastine, did not show cooperativity. Overt toxicity was not evident in any of these trials. Doxorubicin itself produced tumour regression in the MMTV-*Neu* model at higher doses, but this was associated with severe side effects (slumping and inactivity of treated mice). At a lower dose of doxorubicin, 1MT enhanced regression without increasing evident toxicity. Of two signal transduction inhibitors tested, rapamycin showed no evidence of cooperativity while a farnesyltransferase inhibitor (FTI) did. This latter observation might be explained by accumulating evidence that anticancer effects of FTIs appear not to be mediated through inhibition of Ras signalling, as originally thought, and that a DNA damage mechanism has instead recently been invoked [110]. As expected, the iron chelator tetrathiomolybdate, which has been reported to block angiogenesis, also

showed no cooperativity with 1MT. The implications of these findings are striking as they suggest, perhaps counterintuitively, that modulating immunity with small-molecule inhibitors of IDO in conjunction with conventional cytotoxic chemotherapeutic drug-based treatments might have clinical relevance. This notion that immunotherapy and chemotherapy can be effectively combined to destroy cancer cells is consistent with other preclinical studies that have focused on different immunotherapeutic principles [106,107,111] and the idea that immunotherapy might cooperate with chemotherapy appears to be gaining currency as evidenced by the appearance of review articles addressing this topic [112-114].

3. Indoleamine 2,3-dioxygenase inhibitors: chemistry and pharmacology

3.1 Structural classes of IDO inhibitory molecules

There are only a small collection of reports describing inhibition studies of indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.17). Not surprisingly, the studies have focused primarily on derivatives of tryptophan (Trp) and structurally related heterocycles like β-carboline, despite the reported [23,115,116] promiscuity of IDO compared with the related tryptophan 2,3-dioxygenase (TDO2, EC 1.13.11.11). Both competitive and noncompetitive inhibitors of IDO have been identified. To date, competitive inhibitors are primarily derivatives of Trp, whereas noncompetitive inhibitors are derivatives of β-carboline.

3.1.1 Competitive inhibitors

Substrate inhibition with high concentrations (> 0.2 mM) of L-Trp was reported [117,118] during early enzymological studies; therefore, it is not surprising that Trp derivatives have been extensively studied. Derivatisation of the Trp structure has occurred in three areas: substitution of the indole ring, modification of the amino acid side chain, and modifications of the indole ring.

3.1.1.1 Tryptophan indole ring substitutions

Substitution of the indole ring of Trp (**Figure 1**) has afforded the most commonly used inhibitor of IDO: 1-methyl-Trp (**1**) [63]. A racemic mixture was originally used by Munn and co-workers in their seminal study of the fetal survival paradox, [59] but subsequent studies [119] with isolated IDO have revealed a slight preference for the natural L (S) isomer of **1** (the more precise Cahn-Ingold-Prelog system of configurational assignment will subsequently be used in preference to the historic D,L system). Stereochemical preference for the natural isomer was also reported with the 6-nitro derivative **18** (**Table 1**). On the other hand, some cellular studies [76.93,94] demonstrate greater activity for the R (D) isomer of **1** (1-MT). Given the more complex nature of cellular studies, IDO inhibitory activity may not be the primary reason for the better activity of the R isomer of **1**. Nevertheless, based on these conflicting results, future inhibition studies should carefully consider both stereoisomers of Trp analogues.

Table 1 comprehensively summarises the range of substituents that have been tested on the indole ring of Trp. The seven most potent compounds based on the reported inhibition data are the five monosubstituted derivatives, 1-methyl (**1**), 5-bromo (**9**), 6-fluoro (**17**), 6-nitro (**18,** S isomer), 7-fluoro (**20**), and the two difluorinated derivatives, 4,7-difluoro (**8**) and 5,7-difluoro (**15**). Excluding the 1-methyl derivative, the six others are electron withdrawing groups [120-122]. Because the three proposed mechanisms [123,124] for IDO catalysis of the conversion of Trp to N-formyl-kynurenine all begin with nucleophilic attack of the pyrrole ring of Trp, electron withdrawing groups on the indole ring would make this step less favourable and slower. Nevertheless, the activity data in **Table 1** indicates that the 5-bromo (**9**) and the 6-fluoro (**17**) derivatives still undergo oxidation, therefore some of these compounds still behave as substrates despite their deactivating substitution.

Several compounds, notably the 5-bromo (**9**) and 2-hydroxy (**6**) derivatives, have significantly different IDO inhibition values reported by different sources. Some of the variability may be due to the different IDO sources and different assay conditions used in different studies. Peterson and co-workers extracted IDO from human monocyte/macrophage cells induced by IFN-γ [119]. They monitored IDO activity by detecting kynurenine product with a radioimmunoassay or HPLC assay. Southan and co-workers used recombinant human IDO, expressed in and purified from *E. coli* [125]. They followed IDO activity with a spectrophotometric assay that detected an imine derivative of kynurenine. Several inhibitors reported in later tables were evaluated against IDO isolated from rabbit small intestine using two different detection methods [63,126]. Despite these differences, several compounds show striking consistency, for example, **1** and **13** (**Table 1**) and **39** (**Table 2**).

Several electron-releasing substituents in **Table 1** are very active as substrates and are oxidised by IDO: 4-methyl (**7**), 5-methyl (**10**), 5-methoxy (**11**), 5-hydroxy (**13**) and 6-methyl (**16**). One derivative (5-methyl, **10**) is more active than L-Trp. This result is consistent with the mechanistic rational and the outcome described for the electron withdrawing substituents. Electron releasing substituents would be expected to make the indole ring more nucleophilic leading to a faster initial reaction with the oxygen species at the active site.

The 1-methyl derivative **1** defies the trend seen with substituents on the benzene portion of the indole ring. The proposed mechanisms [123,124] for IDO involving pyrrole electron donation, actually initiate the nucleophilic attack with deprotonation of the N-1 hydrogen of Trp. Without a hydrogen, 1MT prevents the deprotonation from occurring. Similar inhibition is seen with benzofuran (**48**, **Table 3**) and benzothiophene (**49**) analogues of Trp (vide infra). However, there is a limited amount of space in the active site to accommodate N-1 groups as the 1-ethyl (**2**) and 1-phenyl-sulfonyl (**3**) derivatives exhibited only weak inhibitory activity.

Table 1. Tryptophan derivatives with indole ring substitution.

Compound	Indole ring substition	Stereochemistry at α-position	Inhibition data (%)*	Activity data (%)‡	Reference
1	1-CH_3	S (L)	52.3 (62.9)§; K_i = 34 μM§		[119]
1	1-CH_3	R,S	26; K_i = 6.6 μM¶	7	[125]
1	1-CH_3	R (D)	5.7 (11.6)§		[119]
2	1-CH_2CH_3	S	13.5 (9.9)§		[119]
3	1-SO_2Ph, 6-OCH_3	R	3.2 (28.4)§		[119]
4	2-Cl	S	20	33	[125]
5	2-Br	S	11	21	[125]
6	2-OH	S	30	4	[125]
6	2-OH	R,S	-38.4 (-43.3)§		[119]
7	4-CH_3	R,S	26	33	[125]
8	4-F, 7-F	S	K_i = 40 μM		[123]
9	5-Br	R,S	0c		[119]
9	5-Br	R,S	56	36	[125]
10	5-CH_3	R,S	6	123	[125]
11	5-OCH_3	R,S	35	70	[125]
12	5-OCH_2Ph	R,S	2	1	[125]
13	5-OH	S	12	59	[125]
13	5-OH	S	14§		[119]
14	5-F	R,S	32	46	[125]
15	5-F, 7-F	S	K_i = 24 μM		[123]
16	6-CH_3	R,S	20	72	[125]
17	6-F	R,S	54	38	[125]
18	6-NO_2	S	52	2	[125]
18	6-NO_2	R	7	0	[125]
19	7-CH_3	R,S	36	18	[125]
20	7-F	S	K_i = 37 μM		[123]

* Unless otherwise stated, inhibition data are reported as 100 minus percentage of tryptophan metabolised in an *in vitro* competitive inhibition assay with 1 mM of inhibitor. Per cent in parenthesis indicates inhibition data with 2 h preincubation of inhibitor with indoleamine 2,3-dioxygenase.

‡Per cent compound oxidized relative to L-tryptophan.

§100 μM inhibitor concentration used in inhibition assay.

¶K_i determined at pH 8.0 in [63].

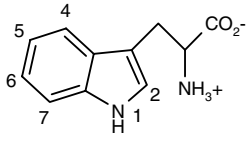

Figure 1. Structure of the tryptophan amino acid, including the indole ring. Substitution positions are labelled 1 – 7.

Indole ring substitution of Trp derivatives has been extensively explored. Nevertheless, the use of multiple substituents is a strategy that might yield more active inhibitors. Excluding compounds **3**, **8** and **15**, there are few compounds with multiplte substituents that have been synthesised and tested. The synthetic challenge posed by polysubstituted indoles is probably one reason that these examples are limited. Another limitation would appear to be the space available in the indole binding region of the active site as seen in the weak activity and inhibition with **12**. Despite these limitations, it is clear that a range of substituents has been accommodated and therefore combinations of these might afford synergistic inhibition.

Table 2. Tryptophan side chain modifications.

Compound	R = *	Stereochemistry at α-position	Inhibition data (%)‡	Activity data (%)§	Reference
21	$-CH_2CH_2NH_2$		28	32	[125]
22	$-CH_2CH_2NH_2$; {5-OCH_3}		-43.9¶		[119]
23	$-CH_2CH_2NH_2$; {2-CO_2H}		16.3 (17.9)¶		[119]
24	$-CH_2CH_2NH_2$; {2-CO_2H, 5-OCH_3}		10.8 (3.4)¶		[119]
25	$-CH_2CH_2CO_2H$		0	8	[125]
26	$-CH_2C(CH_3)(NH_2)CO_2H$	R,S	1	35	[125]
27	$-CH_2CH(NHCH_3)CO_2H$	S	33	21	[125]
28	$-CH_2CH(NHCOCH_3)CO_2H$	S	7	3	[125]
29	$-CH_2CH(NH_2)CO_2CH_3$	S	30	15	[125]
30	$-CH_2CH(NH_2)CO_2CH_2CH_3$	S	7	14	[125]
31	$-CH_2CH(OH)CO_2H$	R,S	9.7 (1.4)¶		[119]
32	$-CH_2N(CH_3)_2$		-6.6¶		[119]
33	$-CH_2CN$		3.5¶		[119]
34	$-C(O)NH_2$; {5-OH}		0¶		[119]
35	-CHO		4.4¶		[119]
36	$-CH = CHCO_2H$		2.5 (3.2)¶		[119]
37	$-CH = CHCO_2CH(CH_3)_2$		15.2 (11.6)¶		[119]
38	-(E)-CH = CH-(3-pyridinyl); {6-F}		0		[127]
39	$-CH(CH_3)CH(NH_2)CO_2H$	α-S,β-S; α-R, β-R	0.0 (-2.7)¶		[119]
39	$-CH(CH_3)CH(NH_2)CO_2H$	α-S,β-R; α-R, β-S	9.8 (3.6)¶		[119]
39	$-CH(CH_3)CH(NH_2)CO_2H$	R,S	7	32	[125]
40	$-CH_2$-5′-(3′-methyl-2′-thioxo-4′-imidazolinone)	R,S	K_i = 11.4 μM		[90]
41	$-CH_2CH(NH_2)CO$-(S)-Trp	S	K_i = 147 μM		[119]

* Additional indole substituents are added in brackets.

‡ Unless otherwise stated, inhibition data are reported as 100 minus percentage of tryptophan metabolised in an *in vitro* competitive inhibition assay with 1 mM of inhibitor. Per cent in parenthesis indicates inhibition data with 2 h preincubation of inhibitor with indoleamine 2,3-dioxygenase.

§ Per cent compound oxidised relative to L-tryptophan.

¶ 100 μM inhibitor concentration used in inhibition assay.

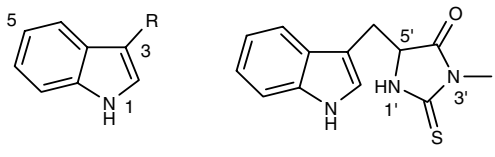

Figure 2. Tryptophan side chain and modification in compound 40.

Unlike the β-carboline derivatives (vide infra), there has been no indication of slow-binding inhibition from Trp derivatives; the preincubation inhibitory data in **Tables 1 – 3** does not substantially differ from the percentage inhibition found in standard competition assays.

3.1.1.2 Tryptophan side chain modifications

A range of Trp side chain modifications have been explored as illustrated in **Table 2**. However, relatively few of these have afforded compounds with promising inhibition. Modest inhibition was realised with the addition of a methyl group to either the α-amine (**27**) or the α-acid (**29**). One notable derivative with interesting activity and a novel structure is the thiohydantoin derivative **40** (**Figure 2**) [90]. Further modification of the thiohydantoin ring might provide even more potent inhibitors.

3.1.1.3 Indole ring modifications

Modifications of the indole ring have afforded a few novel competitive inhibitors (**Table 3**). Most notable amongst this group are the benzofuran (**48**) and benzothiophene (**49**) derivatives described earlier. These two compounds, like

Table 3. Indole ring modifications of tryptophan.

Compound	Side chain (CH_2-)	Stereochemistry at α-position	Inhibition data (%)*	Reference
42	3-(1H-Indazolyl)-	R,S	0.0	[119]
43	3-(7-Azaindolyl)-	R,S	-1.6	[119]
44	3-Indolinyl	S	0.4 (3.0)	[119]
44	3-Indolinyl	R	-2.4 (-1.2)	[119]
45	3-Quinolinyl	S	0	[119]
45	3-Quinolinyl	R	0	[119]
46	(2-Amino-phenyl)methyl	S	-0.3	[119]
47	(2-Amino-3-hydroxy-phenyl)methyl	R,S	-0.4	[119]
48	3-Benzofuranyl	R,S	43‡,§	[125]
48	3-Benzofuranyl	R,S	K_i = 25 μM	[63]
49	3-Benzothiophenyl	R,S	16‡,¶	[125]
49	3-Benzothiophenyl	R,S	K_i = 70 μM	[63]
50	1-(1,4-Cyclohexadienyl)	S	K_i = 230 μM	[128]

* Unless otherwise stated, inhibition data are reported as 100 minus percentage of tryptophan metabolised in an *in vitro* competitive inhibition assay with 100 μM of inhibitor. Per cent in parenthesis indicates inhibition data with 2 h preincubation of inhibitor with indoleamine 2,3-dioxygenase.

‡ 1 mM inhibitor concentration used in inhibition assay.

§ 22% of 44 was oxidised by indoleamine 2,3-dioxygenase.

¶ 19% of 45 was oxidised by indoleamine 2,3-dioxygenase.

1-methyl-Trp (**1**), lack an N-1 proton and, therefore, cannot be deprotonated; the initial step in the proposed catalytic mechanism of IDO indole oxidation [123,124]. Attempts at identifying feedback inhibition from subsequent intermediates in the kynurenine pathway failed with the kynurenine analog **46** and the 3-hydroxykynurenine analog **47**. Surprisingly, based on the success of electron withdrawing groups on the benzene portion of the indole (**Table 1**), a π-deficient analog of indole, 7-azaindole (**43**), also failed to demonstrate inhibitory activity. Similarly, modifications of either the pyrrole portion of the indole ring, i.e., reduction (**44**) or incorporation of another nitrogen (**42**), also failed to afford inhibition. The majority of the data from **Table 3** indicates that the indole ring is almost essential for the creation of a competitive inhibitor.

3.1.1.4 Miscellaneous structures

A small selection (**Table 4**) of structures unrelated to Trp has been tested for competitive inhibition. Similar to the modified indole ring structures in **Table 3**, the majority of the structures have not shown any inhibitory activity. Feedback inhibition was not detected with kynurenic acid (**54**) or quinolinic acid (**57**), nor was inhibition seen with the structurally related analogs **53**, **55** and **56**. Two interesting exceptions were discovered with **52** and **58**. 3-Amino-2-naphthoic acid (**52**) is an analog of anthranilic acid, an intermediate in the aromatic pathway of Trp metabolism. Although assay differences preclude direct comparisons of the potency of IDO inhibitors, compound **52** is one of the most potent inhibitors yet reported in the literature. It is clearly one of the most interesting lead compounds, notwithstanding the synthetic challenge of constructing 3-amino-2-naphthoic acid analogs. A second unique inhibitor was pyrrolidine dithiocarbamate (**58**) [129]. This antioxidant demonstrated notable inhibitory activity of IDO generated from IFN-γ treatment of human monocyte-derived macrophages. It is possible that the sulfur of the dithiocarbamate is binding to the haem iron in the active site of IDO. This binding mode would be consistent with the well-known affinity of sulfur for iron in biological systems (e.g., ferrodoxin).

3.1.2 Noncompetitive inhibitors

The first class of structures exhibiting IDO inhibition was a series of β-carboline structures reported in 1984 [126]. Initially, they were reported to exhibit uncompetitive inhibition but β-carboline (**59**), also known as norharman, was subsequently reclassified as a noncompetitive inhibitor [130]. β-Carboline derivatives (**Table 5**, **Figure 3**) continue to be the most common type of noncompetitive inhibitor, but there are three novel structures (**Table 6**, **Figure 4**) that have also been reported [131].

3.1.2.1 β-Carboline derivatives

Modifications to the β-carboline structure have occurred in both the pyridine and the benzene rings. The pyridine ring has been reduced and substituted at C-1 and C-3. The benzene ring has been substituted at C-6 and C-7. There are still many positions of the β-carboline structure that have not

Table 4. Other compounds tested for competitive inhibition.

Compound	Structure	Inhibition data (%)*	Reference
51	1-Amino-2-naphthoic acid	-2.0 (11.2)	[119]
52	3-Amino-2-naphthoic acid	74.2 (75.2)	[119]
53	3-Quinolinecarboxylic acid	-2.6	[119]
54	4-Hydroxy-2-quinolinecarboxylic acid	1.1	[119]
55	4,8-Dihydroxy-2-quinolinecarboxylic acid	2.9	[119]
56	2-Picolinic acid	1.5	[119]
57	Quinolinic acid	6.8	[119]
58	Pyrrolidine dithiocarbamate	44‡; IC_{50} = 6.5-12.5 μM	[129]

* Unless otherwise stated, inhibition data are reported as 100 minus percentage of tryptophan metabolised in an *in vitro* competitive inhibition assay with 100 μM of inhibitor. Per cent in parenthesis indicates inhibition data with 2 h preincubation of inhibitor with indoleamine 2,3-dioxygenase.
‡ 125 mM inhibitor concentration used in inhibition assay.

been explored. The most potent IDO inhibitors have larger alkyl substituents in the C-3-position (e.g., **74** and **76**). There appears to be a hydrophobic pocket in the active site capable of accommodating these alkyl groups. Fluorine and the isothiocyanate group were present in several potent C-6 substituted β-carboline derivatives (e.g., **68**, **76** and **77**).

As noncompetitive inhibitors, β-carboline derivatives do not compete for the same active site location as Trp or other indoleamine substrates. Nevertheless, there is experimental evidence that indicates that β-carboline **59** binds directly to the haem iron at the active site as a nitrogen ligand and competes with oxygen for binding at the active site iron [130]. Sono has determined that the β-carboline occupies another binding site close to the L-Trp binding region and he hypothesises that this space may be available for a natural cofactor or a regulator of the enzyme [25]. Interestingly, several of the β-carboline inhibitors (i.e., **61**, **64** and **66**) demonstrated considerably greater potency on preincubation with IDO. This is suggestive of slow-binding inhibition and may indicate these inhibitors need time to settle into the second binding pocket near the haem iron. One important liability of β-carboline derivatives is the reported neuroactivity of these structures as benzodiazepine receptor ligands [132-135]. In fact, many previous IDO inhibitor studies were focused on developing treatments for neurological disorders (e.g., excitotoxic brain lesions) where penetration of the central nervous system may have been necessary. However, an IDO inhibitor that was able to penetrate the CNS could cause problematic side effects in cancer therapy.

3.1.2.2 Miscellaneous structures

A small group of other compounds have been discovered to be noncompetitive inhibitors. Although limited in number, these structures provide some unique and potent structural leads. 4-phenylimidazole (**87**) is believed to bind to the haem iron, similar to β-carboline (**59**) [25]. It seems possible that brassilexin (**89**) may also bind to the haem iron through the sulfur of the isothiazole ring.

3.1.3 Summation

Although a selection of compounds have been investigated for IDO inhibition, submicromolar inhibition has not yet been achieved. A few unique structures have been discovered to have IDO inhibitory activity, nonetheless the majority of the most active structures contain the indole system or resemble L-Trp. Clearly, one important goal in the development of IDO inhibition as a cancer therapy will be to discover more potent inhibitors, and it seems that a diversification of IDO inhibitor structures may be necessary to achieve this goal.

3.2 Indoleamine 2,3-dioxygenase inhibitors *in vivo*

3.2.1 Administration of IDO inhibitors

Until recently, 1MT has been the only compound evaluated as an IDO inhibitor *in vivo*. The means of delivery used in the majority of studies has been the same as that used in the original allogeneic conceptus rejection study [59]. This involves encapsulating the 1MT compound in a polymer matrix. The pellets (as prepared by Innovative Research, Inc.) are claimed to provide a steady-state release rate of 10 mg/day, although no data directly demonstrating this has been provided. During the course of tumour treatment studies, the authors have evaluated the serum levels of 1MT achieved with subcutaneous pellet implants. Two 140 mg pellets per mouse resulted in a steady-state level of 1MT in the serum of 100 μM within 24 h after implantation. At the published compound release rate these pellets were expected to maintain a constant level of serum 1MT for a period of 2 weeks. The authors found, however, that this steady-state level of 1MT was maintained for only 5 – 7 days postimplantation after which time serum 1MT rapidly dropped to negligible levels. An alternative procedure for delivering 1MT in drinking water has been reported in one study [85]. However, this method of compound delivery provides only limited control over the frequency and amount of each administered dose and can reportedly result in dehydration of the animals.

The authors' own recent work has determined that efficacious administration of 1MT can be achieved by oral gavage

Table 5. β-Carboline ring substitution compounds.

Compound	β-Carboline ring substitution	Inhibition data (%)*	Reference
59	None	50.3 (57.0); K_i = 178 μM	[131]
60	3-OCH_2CH_3	5.5 (21.2)	[131]
61	3-$OCH_2CH_2CH_3$	16.7 (76.7) ; K_i = 98.0 μM	[131]
62	3- OCH_2CH_2OH	6.7 (11.0)	[131]
63	3-CO_2t-Bu	7.0 (7.2)	[131]
64	3-$C(O)CH_2CH_2CH_3$	-4.1 (44.9)	[131]
65	3-NH_2	0.9 (-19.4)	[131]
66	3-N = C = S	26.7 (86.1)	[131]
67	3-OH	30.1 (-5.3)	[131]
68	3-CO_2CH_3, 6-F	40.4 (49.2); K_i = 7.4 μM	[131]
69	3-CO_2CH_3, 6-Br	-4.9 (13.4)	[131]
70	3-CO_2H	K_i = 40.6 μM	[131]
71	3-CO_2CH_3	K_i = 259 μM	[131]
72	3-$CO_2CH_2CH_2CH_3$	K_i = 98.0 μM	[131]
73	3-$CO_2C(CH_3)_3$	K_i = 89.7 μM	[131]
74	3-$CH_2CH_2CH_2CH_3$	K_i = 3.3 μM	[131]
75	3-NO_2	K_i = 37.5 μM	[131]
76	3-$CO_2CH_2CH_3$, 6-F	K_i = 21.0 μM	[131]
77	3-CO_2CH_3, 6-N = C = S	K_i = 8.5 μM	[131]
78	1-CH_3, 7-OCH_3	10‡	[126]
79	1-CH_3, 2-O, 7-OCH_3	46§	[126]
80	1-CH_3, 7-OH	-11‡	[126]
81	1-CH_3	-13‡	[126]
82	1- CO_2CH_3, 7- OCH_3	25‡	[126]
83	1-CH_3, 7-OCH_3, 3,4-dihydro	4‡	[126]
84	1-CH_3, 7-OH, 3,4-dihydro	21‡	[126]
85	1,2,3,4-tetrahydro	0§	[126]
86	1-OH, 7-OCH_3, 3,4-dihydro	-13§	[126]

* Unless otherwise stated, inhibition data are reported as 100 minus percentage of tryptophan metabolised in an *in vitro* competitive inhibition assay with 100 μM of inhibitor. Per cent in parenthesis indicates inhibition data with 2 h preincubation of inhibitor with indoleamine 2,3-dioxygenase.

‡ 2 mM inhibitor concentration used in inhibition assay with rabbit intestine indoleamine 2,3-dioxygenase.

§ 1 mM inhibitor concentration used in inhibition assay with rabbit intestine indoleamine 2,3-dioxygenase.

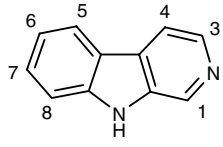

Figure 3. Structure of the β-carboline ring. Substitution positions are labelled 1 – 8.

bolus dosing of a bead-milled suspension prepared in methocel/tween (0.5% methylcellulose/1% tween 80). Single-dose intravenous and oral pharmacokinetic profiles of 1MT administered at 40 mg/kg in mice have been published [90]. Based on the favourable oral bioavailability and relatively slow clearance of 1MT, we have developed an oral dosing schedule that, in combination with paclitaxel administration, produces regression of MMTV-*Neu* tumours (AJ Muller, J DuHadaway, E Sutanto-Ward and GC Prendergast, manuscript in preparation). The ability to effectively deliver 1MT in a controlled manner through oral administration will greatly facilitate further acquisition of preclinical data regarding dosing and scheduling parameters for the development of an optimized combinatorial treatment protocol that will provide a necessary contextual framework within which to evaluate new, more effective, IDO inhibitors as they are identified.

Table 6. Other compounds demonstrating noncompetitive inhibition.

Compound	Structure	Inhibition data (%)*	Reference
87	4-Phenylimidazole	K_i = 4.4 μM	[130]
88	Camalexin	21.3	[131]
89	Brassilexin	K_i = 5.4 μM	[131]

* Unless otherwise stated, inhibition data are reported as 100 minus percentage of tryptophan metabolised in an *in vitro* competitive inhibition assay with 100 μM of inhibitor.

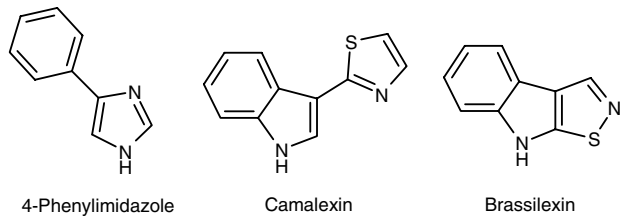

Figure 4. Structures of other compounds which demonstrate noncompetitive inhibition of indoleamine 2,3-dioxygenase.

As noted in Secton 3.1.1.2, the thiohydantoin side chain modification of tryptophan produced a compound (MTH-Trp, **40**) with threefold higher potency against purified human IDO enzyme than 1MT. The differential was even more pronounced in a cell-based assay, in which MTH-Trp was demonstrated to be ~ 20-times more potent than 1MT (EC_{50} = 12 .5 μM for MTH-Trp versus > 200 μM for 1MT [90]). MTH-Trp delivered in time release pellet format in combination with paclitaxel was more effective than a comparable amount of 1MT at producing regression of tumours in the MMTV-*Neu* model [90]. This demonstration that a structurally distinct IDO inhibitor exhibits a biological effect comparable to that of 1MT bolsters the argument that the proposed mechanism of action is correct. MTH-Trp efficacy by oral gavage delivery has not been evaluated because pharmacokinetic analysis has revealed that MTH-Trp, unlike 1MT, is rapidly cleared from the bloodstream [90]. Thus, it appears unlikely that the efficacy achieved through oral gavage bolus dosing of 1MT in combination with paclitaxel can be replicated with MTH-Trp.

3.2.2 Serum kynurenine as a biomarker for IDO activity

Studies have reported the functional consequences of IDO inhibitor treatment without assessing IDO inhibition *in vivo* or correlating IDO inhibition with the observed biological consequences. This raises the legitimate concern of 'off target' effects (e.g., through interaction with other enzymes that bind tryptophan). The degradation of tryptophan by IDO produces *N*-formylkynurenine. This, in turn, is rapidly converted to kynurenine due to high levels of kynurenine formamidase activity, the specific activity of which far exceeds that of IDO. However, other than liver and kidney, most tissues examined exhibit no significant activity associated with other key enzymes on the kynurenine pathway. Rather than being further metabolised in these tissues, kynurenine instead appears to pool in both tissue and blood spaces and systemic induction of IDO in mice by LPS correlated, in large part, with changes in plasma kynurenine levels [44]. Levels of kynurenine as well as tryptophan in the serum can be simultaneously measured by HPLC-based analysis [136-138]. The authors are developing this procedure for use as a pharmacodynamic assay to evaluate inhibition of IDO *in vivo* by compounds administered to mice. Intraperitoneal administration of bacterial LPS induces IDO activity in a variety of tissues resulting in the production of kynurenine and its release into the bloodstream. Peak kynurenine levels are reached one day after LPS administration [44,50]. The serum kynurenine pool is rapidly turned over with a half-life of < 10 minutes [44,139], so pre-existing kynurenine should not unduly mask the impact of IDO inhibitor treatment on kynurenine production. The serum level of compound being tested for IDO inhibitory activity can also be determined by high-performance liquid chromatography analysis of the same sample, permitting concurrent collection of pharmacokinetic data from a single experiment.

In human patients, determination of the serum kynurenine to tryptophan ratio has been performed in several studies to estimate IDO activity independent of baseline tryptophan levels, which can be influenced to some extent by dietary tryptophan uptake [140,141]. Measuring tryptophan and kynurenine levels in plasma or serum is much less invasive than enzyme activity tests performed on tissue samples. Thus, the serum kynurenine to tryptophan ratio can serve as a useful *in vivo* biomarker for evaluating IDO inhibitors in the clinic. The serum kynurenine to tryptophan ratio also correlates closely with neopterin concentrations for a variety of diseases [142]. IFN-γ stimulates guanosine triphosphate cyclohydrolase I, the key enzyme for pteridine production, and human monocytes/macrophages release large amounts of neopterin in response to IFN-γ stimulation [143]. Therefore, neopterin might serve as a valuable companion marker that responds to conditions that induce IDO activity, but is insensitive to direct IDO inhibition.

4. Conclusion

From the standpoint of therapeutic development, IDO as a drug target offers a number of appealing features. First, as a single-chain catalytic enzyme with a well-defined biochemistry,

IDO is highly tractable for inhibitor development compared with most other therapeutic targets in cancer. Second, the only other enzyme known to catalyse the same reaction, TDO2, has a much more restricted substrate specificity simplifying the problem of possible 'off target' effects. Third, the medicinal chemistry of indoleamines and indoleamine mimetics is well-developed. Fourth, lead inhibitors exist in 1MT and MTH-Trp, both of which are bioactive and orally bioavailable. These inhibitors may offer tools for clinical validation of the novel combination principle reviewed here. Fifth, an *Indo* gene 'knockout' mouse has been reported to be viable and healthy [73], indicating that inhibitors will be unlikely to produce unmanageable mechanism-based toxicities, although promotion of inflammatory conditions remains a valid concern. Sixth, the combination of tryptophan and kynurenine, (the major substrate and downstream product of the IDO reaction, respectively) provides a useful biomarker for the pharmacodynamic evaluation IDO inhibitors. This analysis can be performed on blood samples, which eliminates the need to obtain and analyse tumour biopsy specimens that can be difficult, expensive, technically challenging and troublesome to obtain from patients on trial. Lastly, small-molecule immunomodulatory agents are likely to offer substantial logistical and cost advantages relative to both biologics and cell-based immunotherapies. IDO has clearly become established as an attractive and tractable target for the development of better small molecule inhibitors for possible use as immunomodulatory adjuvants to standard chemotherapy and with perhaps the potential for even broader applicability for use against diseases characterised by immune suppression.

5. Expert opinion

The successful treatment of advanced, metastatic cancers remains an elusive goal. Anticancer drug development still focuses predominantly on producing compounds that elicit direct cytotoxicity against tumour cells. Cytotoxic chemotherapeutic agents are currently among the most effective agents in the clinic but these compounds do not have favourable safety profiles. Recently, attention has been shifting towards finding drugs that target specific signal transduction pathways, but, at least so far, the therapeutic impact of such agents has been limited. A popular truism in the cancer field today is that specific combinations of targeted agents will have to be tailored to individual tumours based on their genetic makeup. Such balkanization of cancer therapy clearly runs counter to the contemporary managed care culture. This reality, in the authors' opinion, means that the partitioning of cancer patients among a plethora of small niche markets is likely to be a prohibitively expensive proposition.

Strategies aimed at activating antitumour immunity have the potential to be useful against a wide variety of tumours and might be particularly effective for treating disseminated metastases – the overarching problem facing cancer patients. On this basis, many novel anticancer therapies are currently being developed, with the purpose of stimulating immune responses through the use of cytokines, recombinant antibodies directed at tumour cells, antitumour vaccines, or cell-based, tumour-targeted immune therapies [144]. All of these therapies rely on large, complex biologically-based agents or whole cells. Small molecules have clear advantages over biologics in terms of production, delivery and cost. However, few small-molecule agents for stimulating antitumour immunity have been described. In this regard, IDO represents a particularly appealing target. As a classical biochemical enzyme (a rarity amongst cancer targets), IDO is particularly well-suited for pharmacological intervention, there being a wealth of medicinal chemistry knowledge and experience in the successful development of such drugs. A further advantage stems from the determination that IDO inhibitors appear to work most effectively as immunomodulatory adjuncts to conventional chemotherapeutics, as this suggests that use of IDO inhibitors might be successfully added to standard treatment protocols, which should ease their adoption into the clinic.

Like IDO, a number of other factors, upregulated at tumour sites, have been implicated in the establishment of tumoural immune escape including; transforming growth factor B, IL-10, prostaglandin E_2, Fas, tumour necrosis factor-related apoptosis inducing ligand (TRAIL) and receptor-binding cancer antigen expressed on SiSo cells (RCAS)-1 to name a few. Why then focus on IDO? In large measure, it has been a case of the biology leading the way. Many of the same mechanisms implicated in tumoural immune escape are also posited to protect the developing fetus. However, the 1MT experiment [59] (which the authors have reproduced [90]) is so dramatic, that it strongly implicates IDO as being a particularly powerful mechanism for immune protection. Studying the mechanistic basis of how *Bin1* loss contributes to tumorigenicity has further pointed towards the importance of IDO-mediated immune tolerisation [90]. Re-establishing control over IDO that is lost in *Bin1*-null cells might even be a therapeutic alternative to direct inhibition of IDO activity, though how this might actually be achieved is as yet undetermined. Understanding how Bin1 exerts control over IDO is currently an area of active research.

Autochthonous MMTV-*Neu* tumours were found not to be as dependent on IDO activity for survival as the developing fetus. This was not altogether surprising given the weaker nature of the tumour antigens involved and the greater plasticity of cancer cells in responding to immunological pressure. The MMTV-*Neu* mouse mammary gland tumour model was specifically chosen for study because it is not artificially dependent on IDO for its outgrowth but rather mimics, as closely as possible, breast cancer as it develops in humans. Conceptually it makes sense that the problem of immune escape might have to be attacked on multiple fronts to generate an effective antitumour response. What was perhaps counterintuitive, however, was the finding that standard cytotoxic chemotherapeutic agents show striking cooperativity. Therapies that combine

immunotherapy with cytotoxic chemotherapy have not been widely explored, perhaps because of the assumption that such combinations will work at cross purposes (because cytotoxic chemotherapy kills immune cells that immunotherapy targets for stimulation). This attitude has been challenged recently by a growing number of reports demonstrating striking complementarity between immunotherapeutic and chemotherapeutic strategies [106-108,111]. The authors' findings likewise argue that IDO inhibitor-based immunotherapy can strongly promote antitumour efficacy in combination with chemotherapy. IDO inhibition was found to potentiate the antitumour efficacy of several DNA damaging agents as well as paclitaxel without elevating side effects of these agents. Although the mechanism underlying the observed pattern of cooperation remains to be defined, it is notable that IDO inhibition cooperated with all of the DNA-damaging agents, but none of the antimetabolic agents tested. One interpretation of this pattern of cooperation is that IDO may facilitate tumour survival in response to certain kinds of genotoxic stress, perhaps by attenuating the immune response elicited by cells that display certain types of DNA damage, sustain certain checkpoint responses, or undergo certain kinds of cell death (e.g., apoptosis versus nonapoptotic cell death). A second interpretation is that the chemotherapies that cooperate with IDO inhibition are those that stimulate antitumour immunity in a complementary manner (i.e., combination therapy is really composed of two immunotherapies). Although there is extensive evidence that the efficacy of cytotoxic drugs is based in their ability to directly kill cancer cells, there is also evidence that many of these drugs can also stimulate antitumour immunity. One appealing aspect of this idea is that it addresses what some might view as a paradox of the authors' findings, namely, how a cytotoxic agent that kills immune cells might possibly cooperate with an immune stimulatory agent. In future work, it will be important to rigorously test these two alternatives, for example, by determining whether or not combinatorial efficacy is retained against tumour cells that are resistant to chemotherapy-induced cell death, and whether or not the cytotoxic components of different effective combinations have similar effects on how the immune system responds to tumours despite diversity in cytotoxic mechanisms.

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Marrying Immunotherapy with Chemotherapy: Why Say IDO?

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Abstract

Activation of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) in cancer cells facilitates immune escape. A recent study now shows how small-molecule inhibitors of IDO can be used to leverage the efficacy of traditional chemotherapeutic drugs that are used to treat cancer in the clinic. By promoting antitumor immune responses in combination with cytotoxic chemotherapy, IDO inhibitors may offer a drug-based strategy to more effectively attack systemic cancer. (Cancer Res 2005; 65(18): 8065-8)

Background

During the breakdown in cellular physiology that accompanies malignant tumor development, cancer cells develop certain emblematic characteristics that include inherent cellular properties (cell intrinsic) as well as properties defined through interaction with the host environment (cell extrinsic). Fundamental cell-intrinsic characteristics of cancer cells include immortalization, growth signal self-sufficiency, insensitivity to growth inhibitory signals, and apoptosis resistance, whereas fundamental cell-extrinsic characteristics include the capacity for angiogenesis, invasion, metastasis, and immune escape. Establishment of the importance of immune escape to malignant progression has been relatively recent (1). Indeed, studies of the cell-extrinsic traits of cancer have, in general, tended to lag behind studies of the cell-intrinsic traits, because the former can not be easily evaluated in simple tissue culture systems. Moreover, these processes are generally associated more with epigenetic changes and modifier effects than with mutation of the classically defined oncogene and tumor suppressor pathways that have, until recently, been the major focus of research in molecular cancer biology.

The interactions between developing tumors and the immune system are complex and dynamic. On the one hand, inflammation provides a host of protumorigenic factors and suppression of immune responses can actually promote tumor regression in some model systems (2). On the other hand, cancer cells are also subject to immune surveillance with pressure on tumors to evade or subvert the immune response that tumor antigens should elicit (3). The development of immunotherapeutic strategies has focused predominantly on stimulating or supplementing immune effector cells. It is becoming increasingly apparent, however, that immune tolerance may be dominant in cancer patients and that it will be essential to breach established immune suppressive mechanisms for immunotherapy to be effective (1).

One strategy of immune escape that is used by cancer cells (Fig. 1) has been adapted from a mechanism that normally exists to prevent maternal immune response to paternal fetal antigens that are present during gestation (4). An inescapable consequence of sexual reproduction among histoincompatible individuals is that some means to circumvent maternal immunity must be hardwired into the system to protect the allogeneic fetus. The catabolic enzyme indoleamine-2,3 dioxygenase (IDO; EC 1.13.11.42) has been implicated in providing immune protection to the developing conceptus. IDO catalyzes the initial step in the degradation of tryptophan in the pathway leading to biosynthesis of NAD^+. Activation of IDO in placental trophoblast cells has been proposed to lead to the establishment of immune tolerance through either localized depletion of tryptophan or accumulation of toxic catabolites. This process is immune suppressive because T cells undergoing antigen-dependent activation are exquisitely sensitive to local tryptophan catabolism, which can cause them to arrest in G_1, become anergic, or die (5–7). In a key experiment, treatment of pregnant female mice with 1-methyl-tryptophan, a small-molecule inhibitor of IDO, has been shown to promote T cell–mediated destruction of allogeneic but not syngeneic concepti (4). IDO has also been more generally implicated in CTL-associated protein-4 (CTLA-4)–induced immune tolerance mediated through reverse B7 signaling *in vivo* (8).

Immune Escape in Cancer: Modulation of Indoleamine-2,3 Dioxygenase Expression by Bin1

A connection between elevated urinary tryptophan catabolites and bladder cancer was first reported in the 1950s (9). Since then, elevated levels of IDO-generated catabolites have been associated with a number of malignancies. This phenomenon was initially thought to be a tumoricidal consequence of IFN-γ, which stimulates expression of IDO in cells (10). However, a radical rethinking of the significance of IDO in cancer has been engendered by its implication in the prevention of allogenic conceptus rejection and by the evidence that IDO is overexpressed in most tumors and/or tumor-draining lymph nodes (11–13). How does IDO become deregulated in cancer cells? One possible answer has emerged from studies of a gene called *Bin1*, a cancer suppressive gene that seems to limit cancer to a large extent by limiting immune escape.

Bin1 was initially identified in a two-hybrid screen for c-Myc-interacting proteins (14). Along with the *Bin3* gene, *Bin1* is one of two related genes that are conserved through evolution to yeast and that define a family of adapter proteins characterized by a unique fold termed the BAR domain (14, 15). Frequent loss or attenuation of Bin1 occurs in advanced breast cancer, prostate cancer, melanoma, astrocytoma, neuroblastoma, and colon cancer (16–19).[1] At least 10 different Bin1 splice isoforms exist in mammalian cells of which two are ubiquitously expressed, whereas the remainder are restricted to specific terminally differentiated tissues including neurons and skeletal muscle cells. The different splice isoforms exhibit different patterns of subcellular localization and cancer suppressive activity, arguing that they have different functions. A precedent for BAR adapter proteins with dual trafficking and transcriptional functions

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doi:10.1158/0008-5472.CAN-05-2213

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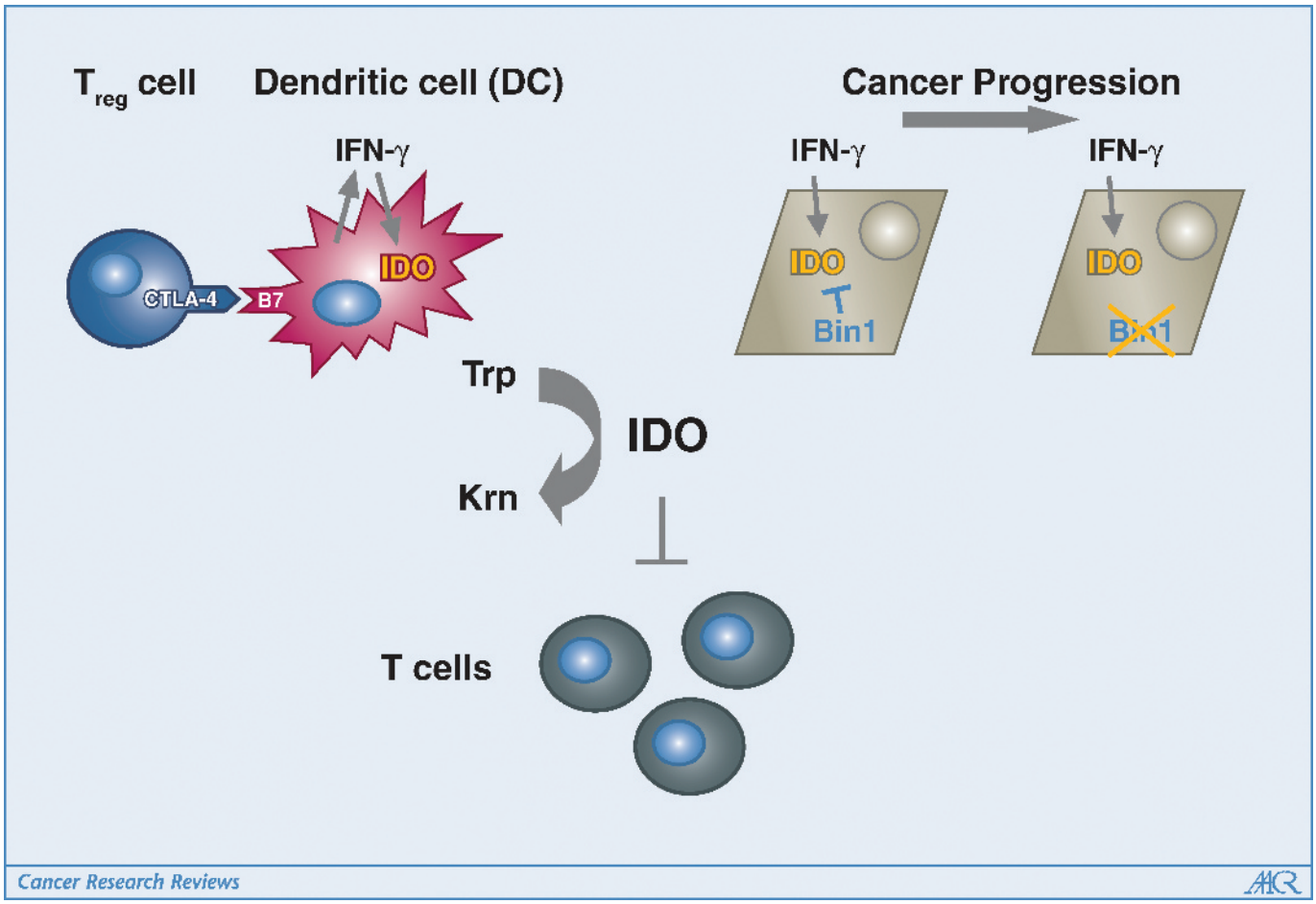

Figure 1. Mechanisms of IDO-induced tumoral immune escape. IDO expression in local immune stroma and directly in tumor cells has been implicated in promoting immune tolerance. IDO is upregulated in antigen-presenting dendritic cells (*DC*) by autocrine IFN-γ released as a result of T_{reg} cell–induced CTLA-4/B7-dependent cell-cell signaling. Local tryptophan catabolism limits the proliferation and survival of T cells that would otherwise be activated by tumor antigens on the APC. This mechanism may operate in tumor-draining lymph nodes. In tumor cells, attenuation of Bin1 leads to superactivation of IDO expression by IFN-γ, directly suppressing activation of T cells in the local tumor environment. Blocking IDO activity systemically with small molecule inhibitors (e.g., 1-methyl-tryptophan) reverses T-cell suppression that occurs as a result of tryptophan catabolism in both settings.

has been established through studies of APPL, a Rab5-binding endosomal protein that translocates to the nucleus upon epidermal growth factor stimulation to associate with the NuRD/MeCP1 nucleosome remodeling and transcriptional repression complex (20). Likewise, the ubiquitously expressed Bin1 splice isoforms, which encode its anticancer properties, have been implicated in both endosomal trafficking and transcriptional repression (21, 22). The possibility that Bin1 adapter proteins may affect pathways leading to the nucleus has garnered additional support based on possible involvement in the trafficking of signal transducer and activator of transcription (STAT) and nuclear factor-κB (NF-κB) transcription factors (23, 24).

Studies aimed at understanding how Bin1 restricts tumor outgrowth identified immune tolerance established through IDO deregulation as a likely mechanistic explanation (25). Deleting the *Bin1* gene from cells resulted in superinduction of IDO gene expression by IFN-γ. *In vitro* transformation of *Bin1*-null and *Bin1*-expressing primary mouse embryo keratinocytes with c-*myc* and mutant *Ras* oncogenes produced cell lines with similar *in vitro* growth properties. However, when these cells were grafted s.c. into syngeneic animals, the *Bin1*-null cells formed large tumors, whereas the *Bin1*-expressing cells formed only indolent nodules. This dichotomy reflected a difference in immune response to the cells, as *Bin1*-expressing cells produced rapidly growing tumors when introduced into either athymic nude mice or syngeneic mice depleted of $CD4^+/CD8^+$ T cells. Treatment of mice with the small-molecule IDO inhibitor 1-methyl-tryptophan suppressed the outgrowth of *Bin1*-null MR KEC tumors in syngeneic mice, but had no effect on tumor growth in mice lacking T cells (either nude mice or immunodepleted syngeneic animals). Taken together, these findings indicated that the deregulation of IDO, which accompanies *Bin1* loss in these cells, promotes tumorigenicity by enabling immune escape. The frequent Bin1 attenuation and IDO overexpression observed in human cancers warrants further evaluation of the relationship between these two events.

Cooperation of Indoleamine-2,3 Dioxygenase Inhibitors with Chemotherapy

The Bin1-IDO studies prompted us to evaluate IDO inhibitors as potential anticancer agents. This effort revealed that immune modulation via IDO inhibition can significantly increase the efficacy of a variety of traditional chemotherapeutic drugs. In several preclinical models of cancer, single-agent therapy with an IDO inhibitor is only marginally efficacious, at best slowing tumor growth

(11, 12, 25). In contrast, regression of established tumors can be achieved by combining an IDO inhibitor with a cytotoxic chemotherapeutic drug (25). In the MMTV-*neu* transgenic mouse model of breast cancer (harboring the c-*neu* proto-oncogene controlled by the mouse mammary tumor virus promoter), which closely resembles human ductal carcinoma *in situ*, established tumors refractory to single-agent therapy underwent regression when enrolled on the combination regimen. This response could not be explained by drug-drug interactions that might raise effective exposure to the cytotoxic agent, and it was dependent on T-cell immunity because depletion of $CD4^+$ T cells abolished the efficacy of the combination therapy. These results offer an initial step in validating IDO as a drug development target in the context of a cytotoxic combination treatment modality.

As a possible drug development target, IDO has a number of appealing features. First, as a single-chain catalytic enzyme with a well-defined biochemistry, IDO is highly tractable for developing small-molecule inhibitors compared with most other therapeutic targets in cancer. Second, the only other enzyme that catalyzes the same reaction, TDO2, has a more restricted expression and substrate specificity, mitigating "off-target" issues posed by novel agents. Third, bioactive and orally bioavailable "lead" inhibitors exist that serve as useful tools for preclinical validation studies. Fourth, an *Indo* gene "knockout" mouse has been reported to be viable and healthy (26), indicating that IDO inhibitors will be unlikely to produce unmanageable mechanism-based toxicities (although promotion of inflammatory conditions would remain a valid concern). Fifth, pharmacodynamic evaluation of IDO inhibitors can be done easily by examining the blood serum levels of tryptophan and kynurenine, the chief substrate and downstream product of the IDO reaction, respectively. Lastly, small-molecule inhibitors of IDO likely offer substantial logistical and cost advantages relative to biological or cell-based therapies that aim at modulating immunity. IDO inhibitors may be useful not only in cancer but also in other pathologic settings, where it is desirable to relieve immune suppression and/or break immune tolerance (e.g., chronic viral infections).

Future Perspective

One general question raised by the work on combining IDO inhibitors with cytoxic agents is how an immunotherapy can effectively enhance the efficacy of chemotherapy. As detailed elsewhere (27), there are at least six critical factors for inducing an antitumor immune response that might be augmented by cytotoxic chemotherapy including antigen threshold, antigen presentation, T-cell response, T-cell traffic, target destruction, and generation of memory. Consensus is lacking as to whether chemotherapy affects immune responsiveness through direct disruption of toleragenic mechanisms or indirectly through tumor cell killing. In some experimental settings, tumor cell killing by cytotoxic agents has been shown to be critical for cooperativity with no evidence of direct effects on cross-presentation by antigen-presenting cells (APC) or on endogenous immune responsiveness (27). The finding that tumor cells killed by alkylating agents such as cyclophosphamide are more effective at activating APCs, when compared with tumor cells killed by antimetabolites or freeze thaw (28), suggests some specificity to this mechanism of immune stimulation. IFN-γ can reportedly sensitize resistant tumor cell lines to apoptosis induction by cytotoxic agents independent of their p53 status (29). In this way, immunotherapy might cooperate with chemotherapy to augment tumor cell killing and indirectly generate additional proinflammatory signals. On the other hand, there is a long history of cyclophosphamide treatment preferentially neutralizing the suppressor arm of the immune system to enhance antitumor responses (30), and such a mechanism of action has been suggested for other cytotoxic agents as well (31). Recently, there has been a growing realization that it is precisely these tolerizing mechanisms that must be overcome for an immunotherapeutic strategy to be successful (1). In this context, both an IDO inhibitor and a cytotoxic agent might be acting as complimentary immunotherapies. Studies have indeed shown that when enhancement of antitumor T-cell responses by immunotherapy with CTLA-4 antibodies (CTLA-4 blockade) was combined with subtherapeutic doses of chemotherapy that shifted the cytokine profile to that of a Th1 response, this potentiated the treatment of established tumors in a mouse model and correlated with enhanced Th1 responsiveness in the treated mice (31). In this context, it is interesting to note that IDO has been proposed to be a downstream effector for the induction of CTLA-4-mediated immune tolerance (8).

IFN-γ may provide a key to understanding how the complex interplay between tumor and stroma is affected by IDO activity and inhibition. A number of reports argue that IFN-γ suppresses tumor outgrowth. Likewise, IDO activity can have antitumor consequences and its up-regulation by IFN-γ may significantly contribute to the negative effect of IFN-γ on tumors (10). These observations seem to run counter to the idea that IDO contributes positively to tumorigenesis, but this interpretation ignores the inherently complex and evolving nature of the interaction between developing tumors and the host immune system. IFN-γ has been directly implicated in the process called immune editing, whereby the immunogenic environment of the host provides positive selection for reduced tumoral immunogenicity (3). Specifically, IFN-γ signaling contributes to an immune-based host environment that suppresses tumor incidence but which can also drive formation of tumors that are more highly aggressive within an immune context (33). At early stages of tumor development, IDO up-regulation by IFN-γ may be detrimental. However, if tumor cells can adapt to the tryptophan poor environment, then keeping IDO under IFN-γ control could give tumor cells the flexibility of turning IDO off and thereby mitigating its negative consequences in the absence of elevated IFN-γ levels that would signal an active Th1 response.

Alternatively, because IDO acts as the rate-limiting enzyme in NAD^+ biosynthesis, one can also envision scenarios in which constitutive expression of IDO in cancer cells is intrinsically beneficial (e.g., under hypoxic conditions that tend to confer drug resistance). Notably, poly(ADP-ribose) polymerase (PARP)–mediated NAD^+ consumption drives "programmed necrosis" independent of the major apoptotic effectors p53, Bax, Bak, and caspases in cancer cells that have become dependent on glycolysis to maintain ATP levels (34). If tumor cells turn on the NAD^+ biosynthesis pathway, they may be able to override sensitization to PARP. In targeting the rate-limiting step for NAD^+ biosynthesis, IDO inhibitors would be expected to cooperate with chemotherapeutic drugs by reestablishing the sensitivity of tumor cells to PARP activation by these drugs. Unlike apoptosis, this necrotic form of cell death is highly proinflammatory potentially incorporating an immune component into the therapeutic response as well. By raising these issues, studies of IDO inhibitor cooperativity with chemotherapy should not only provide insights into the mechanistic basis for this new therapeutic approach but may also afford a deeper understanding of the complex contextual relationship between cancer cells and the multifaceted immune/stromal environment.

Acknowledgments

Received 6/23/2005; accepted 6/24/2005.

Grant support: Sharpe Foundation of the Bryn Mawr Hospital (A.J. Muller), State of Pennsylvania Department of Health CURE/Tobacco Settlement Award (A.J. Muller and G.C. Prendergast), the Department of Defense Breast Cancer Research Program grants BC044350 (A.J. Muller) and BC021133 (G.C. Prendergast), Charlotte Geyer Foundation (G.C. Prendergast), and NIH grant CA109542-01A1 (G.C. Prendergast).

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Drugs of the Future 2005, 30(9): 000-000

CCC: 0377-8282/2005
DOI: 10.1358/dof.2005.030.08.918200

Review Article

A new cancer immunosuppression target: indoleamine 2,3-dioxygenase (IDO). A review of the IDO mechanism, inhibition and therapeutic applications

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CONTENTS

Abstract

Indoleamine 2,3-dioxygenase (IDO) has recently been implicated in tumor immune escape. In particular, IDO undermines a vigorous antitumor immune response by promoting peripheral tolerance, thereby shaping the host environment to be more hospitable to tumor survival and growth. Consequently, the development of potent IDO inhibitors that compromise this toleragenic mechanism is an important therapeutic goal. To assist in the development of more potent IDO inhibitors, the current review presents the proposed catalytic mechanisms of IDO and comprehensively reviews reported IDO inhibitors. Finally, the successful preclinical application of IDO inhibition in a new anticancer modality is described.

Introduction

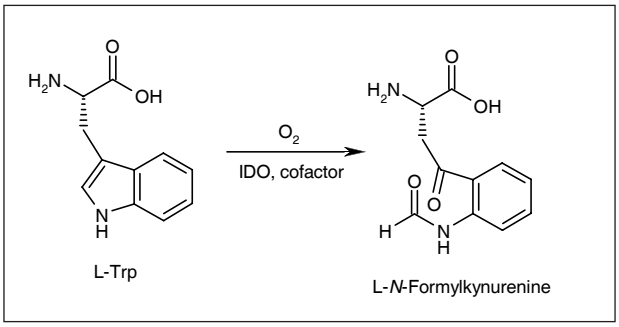
The treatment of advanced (metastatic) cancers is a major clinical challenge. Current regimens involving chemotherapy and other systemic modalities all too often provide only a limited benefit to the approximately 50% of cancer patients in the U.S. and other developed countries who present with advanced disease at diagnosis. Similarly, current regimens ultimately fail patients that relapse with disseminated disease after treatment of their primary tumors. It has long been recognized that tumors display immunogenic tumor antigens yet escape immune destruction, somehow evading or subverting and perhaps even reprogramming the immune system for their own benefit. This phenomenon of "immune escape" is central to tumor cell survival, but its basis is poorly understood (1). An appropriately activated immune system can eradicate cancer, even when it is aggressive and disseminated, but spontaneous occurrences of this are rare. This has prompted the development of numerous peptide- and cell-based anticancer therapies aimed at boosting the immune response (*e.g.*, the administration of cytokines, tumor-associated antigen peptide/vector vaccines, dendritic cell [DC] vaccines and adoptive transfer of tumor antigen-specific effector T-cells expanded *ex vivo* from cancer patients [2-8]). These therapies, which are conceptually based on stimulating components of the immune system that produce an effective response, may not, however, be sufficient to overcome tumor immune escape if pathological immune tolerance is dominant in cancer patients, as has been recently proposed (9).

The enzyme indoleamine 2,3-dioxygenase (IDO), which appears to play a key role in protecting allogeneic conceptus from the maternal immune system, has been implicated in the establishment of pathological immune tolerance by tumors. The physiological role of IDO, which catabolizes the essential amino acid tryptophan, has been defined in large part through the use of the bioavail-

able IDO-inhibitory compound 1-methyltryptophan (1-MT). This review details current thinking regarding the catalytic mechanism of tryptophan degradation by IDO in conjunction with a comprehensive summary of the current literature on small-molecule IDO inhibitors, and concludes with an overview of how IDO-inhibitory compounds might be incorporated into a novel treatment strategy that has the potential to broadly impact standard cancer therapies.

IDO mechanism

IDO is the first and rate-determining step of the kynurenine pathway of L-tryptophan (L-Trp) metabolism. It catalyzes the addition of oxygen across the C-2/C-3 bond of the indole ring in Trp and generates *N*-formylkynurenine (Scheme 1). *In vitro*, methylene blue and ascorbic acid are a necessary reductant to maintain maximum catalytic activity, but *in vivo* a flavin or tetrahydrobiopterin cofactor is believed to serve this role (10, 11).



Scheme 1: IDO reaction.

The rational design and development of IDO inhibitors requires an understanding of the enzyme's mechanism. Although the exact mechanism of IDO remains unknown, important mechanistic research with IDO and non-enzyme-catalyzed oxidation reactions have led to some understanding of the mechanism and several mechanistic proposals. Much of this work was previously described in an earlier review article (11). The current review will summarize the details in the previous review and provide an update on some recent research.

All the proposed mechanisms begin with the binding of O_2 and Trp at the active site of IDO, although the exact order of binding is uncertain. The active form of IDO has the heme iron in the ferrous (Fe^{2+}) oxidation state and, although the enzyme is prone to auto-oxidation, the primary catalytic cycle does not involve redox changes. The ferric (Fe^{3+}) form of IDO is inactive and requires reduction to the ferrous form before catalytic activity is returned. The ferric form is also particularly susceptible to substrate inhibition by Trp (12).

After O_2 and Trp binding in IDO's active site, all the proposed mechanisms proceed through a 3-indolenylperoxy-Fe^{2+} (**1,** Scheme 2). There are three different proposals for the process to reach **1** (Scheme 2) and there are two different proposed mechanisms for the decomposition of **1** to *N*-formylkynurenine (Scheme 3). Intermediate **1** is central to all the proposed mechanisms because the related 3-hydroperoxyindolenine structure (not shown) has been shown to be a competent intermediate in the nonenzymatic oxidation of Trp to *N*-formylkynurenine (13, 14).

*Three mechanisms for the formation of intermediate **1***

1. Ionic mechanism

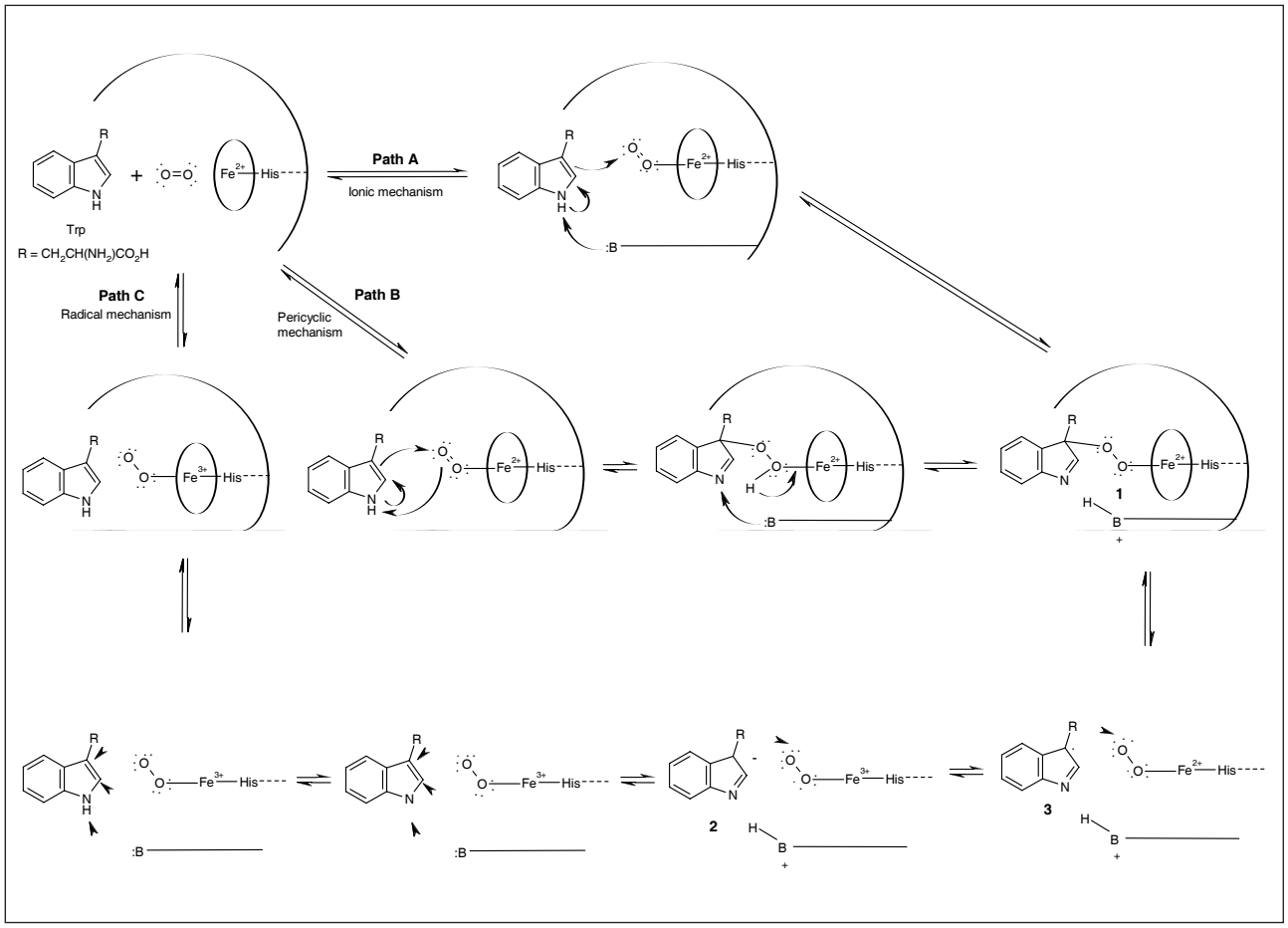
The ionic mechanism (15) has the heme iron serving as a Lewis acid that activates the molecular oxygen (Scheme 2, Path A). Once activated, the electrophilic oxygen undergoes nucleophilic attack by the electron-rich pyrrole portion of the indole to form **1**. Based on substrate and inhibitor studies (16), the N-1 proton of the indole ring of Trp is essential for the oxidation to occur. In the ionic mechanism, it has been proposed that a base in the active site deprotonates the indole to generate a more nucleophilic ring.

2. Pericyclic mechanism

One variant of the ionic mechanism has the distal oxygen of O_2 operating as the basic site (Scheme 2, Path B) (17). Consequently, the reaction is really a pericyclic process and, more precisely, a group transfer reaction similar to an ene reaction (18). Identification of an important basic amino acid in the active site might allow for discrimination between the pericyclic and ionic mechanism. Interestingly, a recent report (19) identified two important amino acids in IDO, His346 and Asp274, through site-directed mutagenesis studies. The authors suggested that the His346 might be the proximal heme iron ligand. The role of the Asp274 is unknown, but the authors suggest it might serve as the distal heme ligand or be important for conformational stability. It is also possible that one of these amino acids is the general base for the deprotonation of the N-1 indole proton.

3. Radical mechanism

The third mechanism (20) (Scheme 2, Path C) involves a one-electron process to reach intermediate **1** and, similar to the ionic mechanism, involves deprotonation of the N-1 indole proton by a base in the active site. However, in the radical mechanism, the indole anion **2** undergoes a one-electron oxidation to generate the intermediate **3**. The two radical structures at the active site can combine to generate **1**.



Scheme 2: Three proposed mechanisms to 3-indolenylperoxy-Fe^{2+} (**1**).

*Two mechanisms for the transformation of **1** to N-formylkynurenine*

1. Criegee-type rearrangement

After formation of the key 3-indolenylperoxy-Fe^{2+} intermediate **1**, two possible pathways are proposed. The first and more likely is a concerted Criegee-type rearrangement to afford the labile cyclic hemiacetal intermediate **4** (Scheme 3, Path D) (15, 21). Simple decomposition of **4** leads to formylkynurenine.

2. Dioxetane retrocycloaddition

Alternatively, **1** may have the distal oxygen add to the C-2 position of the indole to form the dioxetane intermediate **5** (Scheme 3, Path E) (15, 21). A retrocycloaddition of **5** yields *N*-formylkynurenine. Since the formation of the strained intermediate **5** would be thermodynamically unfavorable, this pathway is considered less likely. Furthermore, the highly exothermic decomposition of **5** should lead to light emission, but chemiluminescence has never been detected in the enzyme reaction. Nonenzymatic mechanistic studies also undermine the dioxetane intermediate pathway (22, 23).

The addition of other nucleophiles to the C-2 position of the indole in **1** has also been proposed. Notably, the α-amino group of Trp might add to generate a tricyclic intermediate, 3a-hydroperoxypyrrolo[2,3-*b*]indole derivative (**6**, Fig. 1), which subsequently undergoes conversion to *N*-formylkynurenine with expulsion of the α-amino group. Evidence for the existence of **6** has been found in the nonenzymatic oxidation of Trp (13), but not in the process catalyzed by IDO. Amino acid side-chain residues at the active site have also been proposed to transiently add to the C-2 position of the indole, although no experimental evidence exists to support this idea (23).

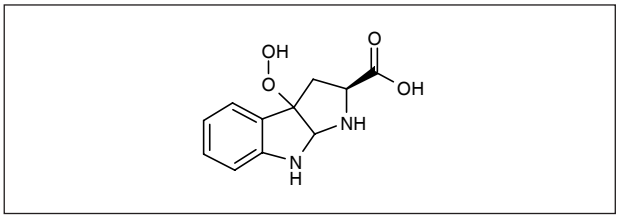
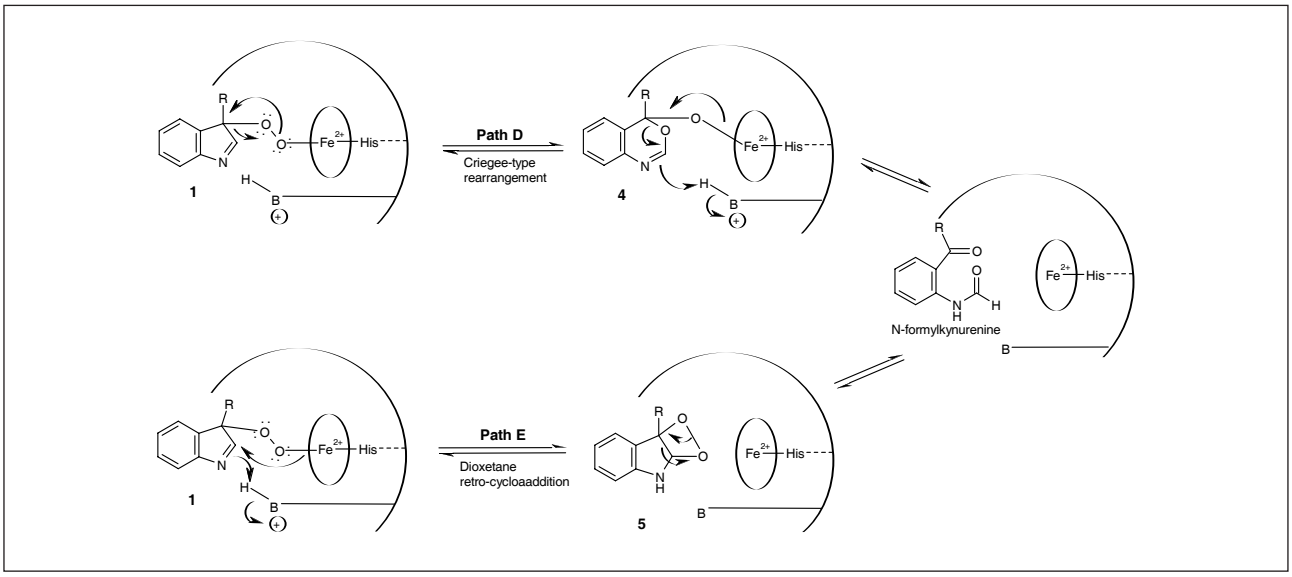


Fig. 1. 3a-Hydroperoxypyrrolo[2,3-*b*]indole derivative (**6**).



Scheme 3: Two proposed mechanisms to *N*-formylkynurenine.

IDO inhibitors: chemistry and pharmacology

Structural classes of IDO-inhibitory molecules

There exists only a small collection of reports describing inhibition studies of indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.17). Not surprisingly, the studies have focused primarily on derivatives of Trp and structurally related heterocycles like β-carboline, despite the reported (24-26) promiscuity of IDO compared to the related tryptophan 2,3-dioxygenase (TDO, EC 1.13.11.11). Both competitive and noncompetitive inhibitors of IDO have been identified. To date, competitive inhibitors are primarily derivatives of Trp, while noncompetitive inhibitors are derivatives of β-carboline; both contain an indole core.

Competitive inhibitors

Substrate inhibition with high concentrations (> 0.2 mM) of L-Trp was reported (12, 27) during early enzymological studies, and therefore it is not surprising that Trp derivatives have been extensively studied. Derivatization of the Trp structure has occurred in three areas: substitution of the indole ring, modification of the amino acid sidechain and modifications of the indole ring.

1. Tryptophan indole ring substitutions

Substitution of the indole ring of Trp has afforded the most commonly used inhibitor of IDO: 1-MT (**7,** Table I) (16). A racemic mixture was originally used by Munn and coworkers in their seminal study of the fetal survival paradox (28), but subsequent studies (29) with isolated IDO have revealed a slight preference for the natural (*S*)-(L) isomer of **7** (the more precise Cahn-Ingold-Prelog system of configurational assignment will subsequently be used in preference to the historic D,L system). Furthermore, the (*S*)-isomer of the natural substrate Trp has 10-50 times smaller K_m values than (*R*)-Trp (30). Stereochemical preference for the natural isomer was also reported with the 6-nitro derivative **24** (Table I). On the other hand, some cellular studies (31-33) demonstrate greater activity for the (*R*)-(D) isomer of **7** (1-MT). Given the more complex nature of cellular studies, IDO-inhibitory activity may not be the primary reason for the better activity of the (*R*)-isomer of **7**. Nevertheless, based on these conflicting results future inhibition studies should carefully consider both stereoisomers of Trp analogues.

Table I comprehensively summarizes the range of substituents that have been tested on the indole ring of Trp. The seven most potent compounds based on the reported inhibition data are the five monosubstituted derivatives, 1-methyl (**7**), 5-bromo (**15**), 6-fluoro (**23**), 6-nitro (**24**), ([*S*]-isomer), 7-fluoro (**26**), and the two difluorinated derivatives 4,7-difluoro (**14**) and 5,7-difluoro (**21**). Excluding the 1-methyl derivative, the other six are electron-withdrawing groups (34-36). Since the proposed mechanisms for IDO-catalyzed conversion of Trp to *N*-formylkynurenine (see above) all begin with electron donation from the pyrrole ring of Trp, electron-withdrawing groups on the indole ring would make this step less favorable and slower. Nevertheless, the activity data in Table I indicates that the 5-bromo (**15**) and the 6-fluoro (**23**) derivatives undergo oxidation; therefore some of these compounds still behave as substrates despite their deactivating substitution.

Several compounds, notably the 5-bromo (**15**) and 2-hydroxy (**12**) derivatives, have significantly different IDO inhibition values reported by different sources. Some of

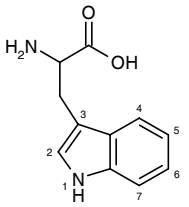


Table I: Trp derivatives with indole ring substitution.

Compound	Indole ring substition	Stereochemistry at α position	Inhibition data (%)[a]	Activity data (%)[b]	Ref.
7	1-CH_3	*S* (L)	52.3 (62.9)[c]; K_i = 34 μM[c]		29
7	1-CH_3	*R,S*	26; K_i = 6.6 μM[d]	7	37
7	1-CH_3	*R,*(D)	5.7 (11.6)[c]		29
8	1-CH_2CH_3	*S*	13.5 (9.9)[c]		29
9	1-SO_2Ph, 6-OCH_3	*R*	3.2 (28.4)[c]		29
10	2-Cl	*S*	20	33	37
11	2-Br	*S*	11	21	37
12	2-OH	*S*	30	4	37
12	2-OH	*R,S*	-38.4 (-43.3[c]		29
13	4-CH_3	*R,S*	26	33	37
14	4-F, 7-F	*S*	K_i = 40 μM		11
15	5-Br	*R,S*	0[c]		29
15	5-Br	*R,S*	56	36	37
16	5-CH_3	*R,S*	6	123	37
17	5-OCH_3	*R,S*	35	70	37
18	5-OCH_2Ph	*R,S*	2	1	37
19	5-OH	*S*	12	59	37
19	5-OH	*S*	14[c]		29
20	5-F	*R,S*	32	46	37
21	5-F, 7-F	*S*	K_i = 24 μM		11
22	6-CH_3	*R,S*	20	72	37
23	6-F	*R,S*	54	38	37
24	6-NO_2	*S*	52	2	37
24	6-NO_2	*R*	7	0	37
25	7-CH_3	*R,S*	36	18	37
26	7-F	*S*	K_i = 37 μM		11

[a] Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 1 mM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.

[b] Percent compound oxidized relative to L-tryptophan.

[c] 100 μM inhibitor concentration used in inhibition assay.

[d] K_i determined at pH 8.0 in reference (16).

the variability may be due to the different IDO sources and assay conditions used in the different studies. Peterson and coworkers extracted IDO from human monocyte/macrophage cells induced by interferon gamma (29). They monitored IDO activity by detecting kynurenine product with a radioimmunoassay or HPLC assay. Southan and coworkers used recombinant human IDO, expressed in and purified from *Escherichia coli* (37). They followed IDO activity with a spectrophotometric assay that detected an imine derivative of kynurenine. Several inhibitors reported in subsequent tables were evaluated against IDO isolated from rabbit small intestine using two different detection methods (16, 38). Despite these differences, several compounds show striking consistency, *i.e.,* **7** and **19** (Table I) and **45** (Table II).

Several electron-releasing substituents in Table I are very active as substrates and are oxidized by IDO: 4-methyl (**13**), 5-methyl (**16**), 5-methoxy (**17**), 5-hydroxy (**19)** and 6-methyl (**22**). One derivative (5-methyl, **16**) is more active than L-Trp. This result is consistent with the mechanistic rationale and the outcome described for the electron -withdrawing substituents. Electron-releasing substituents would be expected to make the indole ring more nucleophilic, leading to a faster initial reaction with the oxygen species at the active site.

The 1-methyl derivative **7** defies the trend seen with substituents on the benzene portion of the indole ring. The proposed mechanisms (see above) for IDO involving pyrrole electron donation actually initiate the reaction with deprotonation of the N-1 hydrogen of Trp. Without a hydrogen, **7** prevents the deprotonation from occurring. Similar inhibition is seen with benzofuran (**54**) and benzothiophene (**55**) analogues of Trp (Table III; see below). However, there is a limited amount of space in the active

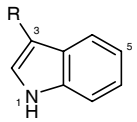


Table II: Trp side-chain modifications.

Compound	R[a]	Stereochemistry at α position	Inhibition data (%)[a]	Activity data (%)[b]	Ref.
27	-$CH_2CH_2NH_2$		28	32	37
28	-$CH_2CH_2NH_2$; {5-OCH_3}		-43.9[d]		29
29	-$CH_2CH_2NH_2$; {2-CO_2H}		16.3 (17.9)[d]		29
30	-$CH_2CH_2NH_2$; {2-CO_2H , 5-OCH_3}		10.8 (3.4)[d]		29
31	-$CH_2CH_2CO_2H$		0	8	37
32	-$CH_2C(CH_3)(NH_2)CO_2H$	*R,S*	1	35	37
33	-$CH_2CH(NHCH_3)CO_2H$	*S*	33	21	37
34	-$CH_2CH(NHCOCH_3)CO_2H$	*S*	7	3	37
35	-$CH_2CH(NH2)CO_2CH_3$	*S*	30	15	37
36	-$CH_2CH(NH_2)CO_2CH_2CH_3$	*S*	7	14	37
37	-$CH_2CH(OH)CO_2H$	*R,S*	9.7 (1.4)[d]		29
38	-$CH_2N(CH_3)_2$		-6.6[d]		29
39	-CH_2CN		3.5 [d]		29
40	-$CONH_2$; {5-OH}		0[d]		29
41	-CHO		4.4[d]		29
42	-$CH=CHCO_2H$		2.5 (3.2)[d]		29
43	-$CH=CHCO_2CH(CH_3)_2$		15.2 (11.6)[d]		29
44	-(*E*)-CH=CH-(3-pyridinyl); {6-F}			0	40
45	-$CH(CH_3)CH(NH_2)CO_2H$	α-*S*,β-*S*;α-*R*,β-*R*	0.0 (-2.7)[d]		29
45	-$CH(CH_3)CH(NH_2)CO_2H$	α-*S*,β-*R*;α-*R*,β-*S*	9.8 (3.6)[d]		29
45	-$CH(CH_3)CH(NH_2)CO_2H$	*R,S*	7	32	37
46	-CH_2-5'-(3'-methyl-2'-thioxo-4'-imidazolinone)	*R,S*	K_i = 11.4 μM		39
47	-$CH_2CH(NH_2)$CO-(*S*)-Trp	*S*	K_i = 147 μM		29

[a] Additional indole substituents are added in brackets.
[b] Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 1 mM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.
[c] Percent compound oxidized relative to L-tryptophan.
[d] 100 μM inhibitor concentration used in inhibition assay.

site to accommodate N-1 groups, as the 1-ethyl (**8**) and 1-phenylsulfonyl (**9**) derivatives exhibited only weak inhibitory activity.

Indole ring substitution of Trp derivatives has been extensively explored; nevertheless, the use of multiple substituents is a strategy that might yield more active inhibitors. Excluding compounds **9**, **14** and **21**, few compounds with multiple substituents have been synthesized and tested. The synthetic challenge posed by polysubstituted indoles is probably one reason that these examples are limited. Another limitation would appear to be the space available in the indole binding region of the active site, as seen in the weak activity and inhibition with **18**. Despite these limitations, it is clear that a range of substituents has been accommodated and therefore combinations of these might afford synergistic inhibition. Unlike the β-carboline derivatives (see below), there has been no indication of slow-binding inhibition from Trp derivatives; the preincubation inhibitory data in Tables I-III do not substantially differ from the percent inhibition found in standard competition assays.

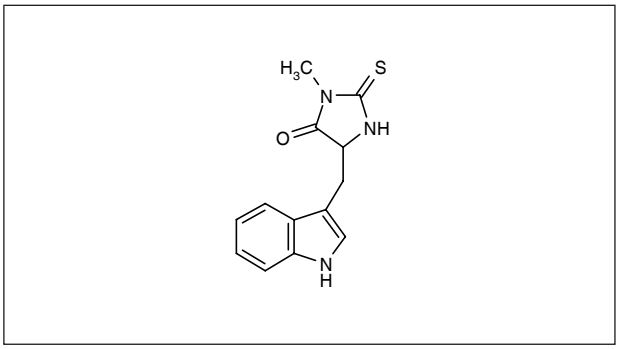


Fig. 2. Compound **46**.

2. Tryptophan side-chain modifications

A range of Trp side-chain modifications have been explored, as illustrated in Table II. However, relatively few of these have afforded compounds with promising inhibition. Modest inhibition was realized with the addition of a methyl group to either the α-amine (**33**) or the α-acid (**35**).

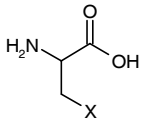


Table III: Indole ring modifications of Trp.

Compound	X	Stereochemistry at α position	Inhibition data (%)[a]	Ref.
48	3-(1*H*-indazolyl)-	R,S	0.0	29
49	3-(7-azaindolyl)-	R,S	-1.6	29
50	3-indolinyl	S	0.4 (3.0)	29
50	3-indolinyl	R	-2.4 (-1.2)	29
51	3-quinolinyl	S	0	29
51	3-quinolinyl	R	0	29
52	(2-aminophenyl)methyl	S	-0.3	29
53	(2-amino-3-hydroxyphenyl)methyl	R,S	-0.4	29
54	3-benzofuranyl	R,S	43[b,c]	37
54	3-benzofuranyl	R,S	K_i = 25 μM	16
55	3-benzothiophenyl	R,S	16[b,d]	37
55	3-benzothiophenyl	R,S	K_i = 70 μM	16
56	1-(1,4-cyclohexadienyl)	S	K_i = 230 μM	41

[a] Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 100 μM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.

[b] 1 mM inhibitor concentration used in inhibition assay.

[c] 22% of **50** was oxidized by IDO.

[d] 19% of **51** was oxidized by IDO.

One notable derivative with interesting activity and a novel structure is the thiohydantoin derivative (**46**) (39). Further modification of the thiohydantoin ring might provide even more potent inhibitors.

3. Indole ring modifications

Modifications of the indole ring have afforded a few novel competitive inhibitors (Table III). Most notable among this group are the benzofuran (**54**) and benzothiophene (**55**) derivatives described earlier. These two compounds, like 1-MT (**7**), lack an N-1 proton and therefore cannot be deprotonated, the initial step in the proposed catalytic mechanism of IDO indole oxidation (11, 17). Attempts at identifying feedback inhibition from subsequent intermediates in the kynurenine pathway failed with the kynurenine analogue (**52**) and the 3-hydroxykynurenine analogue (**53**). Surprisingly, based on the success of electron-withdrawing groups on the benzene portion of the indole (Table I), a pi-deficient analogue of indole, 7-azaindole (**49**), also failed to demonstrate inhibitory activity. Similarly, modifications of either the pyrrole portion of the indole ring, *i.e.*, reduction (**50**) or incorporation of another nitrogen (**48**), also failed to afford inhibition. The majority of the data from Table III indicate that the indole ring is almost essential for the creation of a competitive inhibitor.

4. Miscellaneous structures

A small selection (Table IV) of structures unrelated to Trp have been tested for competitive inhibition. Similar to the modified indole ring structures in Table III, the majority of the structures have not shown any inhibitory activity. Feedback inhibition was not detected with kynurenic acid (**60**) or quinolinic acid (**63**), nor was inhibition seen with the structurally related analogues **59**, **61** and **62**. Two interesting exceptions were discovered with **58** and **64**. 3-Amino-2-naphthoic acid (**58**) is an analogue of anthranilic acid, an intermediate in the aromatic pathway of Trp metabolism. Although assay differences preclude direct comparisons of the potency of IDO inhibitors, compound **58** is one of the most potent inhibitors yet reported in the literature. It is clearly one of the most interesting lead compounds, notwithstanding the synthetic challenge of constructing 3-amino-2-naphthoic acid analogues. A second unique inhibitor was pyrrolidine dithiocarbamate (**64**) (42). This antioxidant demonstrated (notable inhibitory activity against IDO generated from interferon gamma treatment of human monocyte-derived macrophages. It is possible that the sulfur of the dithiocarbamate is binding to the heme iron in the active site of IDO. This binding mode would be consistent with sulfur's well-known affinity for iron in biological systems, *e.g.*, ferrodoxin.

Table IV: Other compounds tested for competitive inhibition.

Compound	Structure	Inhibition data (%)[a]	Ref.
57	1-amino-2-naphthoic acid	-2.0 (11.2)	29
58	3-amino-2-naphthoic acid	74.2 (75.2)	29
59	3-quinolinecarboxylic acid	-2.6	29
60	4-hydroxy-2-quinolinecarboxylic acid	1.1	29
61	4,8-dihydroxy-2-quinolinecarboxylic acid	2.9	29
62	2-picolinic acid	1.5	29
63	quinolinic acid	6.8	29
64	pyrrolidine dithiocarbamate	44[b]; IC_{50} = 6.5-12.5 μM	42

[a] Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 100 μM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.
[b] 125 mM inhibitor concentration used in inhibition assay.

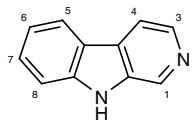


Table V: β-Carboline ring substitution compounds.

Compound	β-Carboline ring substitution	Inhibition data (%)[a]	Ref.
65	none	50.3 (57.0); K_i = 178 μM	44
66	3-OCH_2CH_3	5.5 (21.2)	44
67	3-$OCH_2CH_2CH_3$	16.7 (76.7) ; K_i = 98.0 μM	44
68	3-OCH_2CH_2OH	6.7 (11.0)	44
69	3-CO_2t-Bu	7.0 (7.2); K_i = 89.7 μM	44
70	3-$COCH_2CH_2CH_3$	-4.1 (44.9)	44
71	3-NH_2	0.9 (-19.4)	44
72	3-N=C=S	26.7 (86.1)	44
73	3-OH	30.1 (-5.3)	44
74	3-CO_2CH_3, 6-F	40.4 (49.2); K_i = 7.4 μM	44
75	3-CO_2CH_3, 6-Br	-4.9 (13.4)	44
76	3-CO_2H	K_i = 40.6 μM	44
77	3-CO_2CH_3	K_i = 259 μM	44
78	3-$CO_2CH_2CH_2CH_3$	K_i = 98.0 μM	44
79	3-$CH_2CH_2CH_2CH_3$	K_i= 3.3 ?M	44
80	3-NO_2	K_i = 37.5 μM	44
81	3-$CO_2CH_2CH_3$, 6-F	K_i = 21.0 μM	44
82	3-CO_2CH_3, 6-N=C=S	K_i = 8.5 μM	44
83	1-CH_3, 7-OCH_3	10[b]	38
84	1-CH_3, 2-O, 7-OCH_3	46[c]	38
85	1-CH_3, 7-OH	-11[b]	38
86	1-CH_3	-13[b]	38
87	1-CO_2CH_3, 7- OCH_3	25[b]	38
88	1-CH_3, 7-OCH_3, 3,4-dihydro	4[b]	38
89	1-CH_3, 7-OH, 3,4-dihydro	21[b]	38
90	1,2,3,4-tetrahydro	0[c]	38
91	1-OH, 7-OCH_3, 3,4-dihydro	-13[c]	38

[a] Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 100 μM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.
[b] 2 mM inhibitor concentration used in inhibition assay with rabbit intestine IDO.
[c] 1 mM inhibitor concentration used in inhibition assay with rabbit intestine IDO.

Noncompetitive inhibitors

The first class of structures exhibiting IDO inhibition was a series of β-carboline structures reported in 1984 (38). Initially, they were reported to exhibit uncompetitive inhibition, but β-carboline (**65**), also known as norharman, was subsequently reclassified as a noncompetitive inhibitor (43). β-Carboline derivatives (Table V) continue

to be the most common type of noncompetitive inhibitor, but three novel structures (Table VI) have also been reported (44).

1. β-Carboline derivatives

Modifications to the β-carboline structure have occurred in both the pyridine and the benzene rings. The pyridine ring has been reduced and substituted at C-1 and C-3, and the benzene ring has been substituted at C-6 and C-7. There are still many positions of the β-carboline structure that have not been explored. The most potent IDO inhibitors have larger alkyl substituents in the C-3 position, *e.g.,* **79** and **81**. There appears to be a hydrophobic pocket in the active site capable of accommodating these alkyl groups. Fluorine and the isothiocyanate group were present in several potent C-6-substituted β-carboline derivatives, *e.g.*, **74**, **81** and **82**.

As noncompetitive inhibitors, β-carboline derivatives do not compete for the same active site location as Trp or other indoleamine substrates. Nevertheless, there is experimental evidence that indicates that β-carboline (**65**) binds directly to the heme iron at the active site as a nitrogen ligand and competes with oxygen for binding at the active-site iron (43). Sono has determined that the β-carboline occupies another binding site close to the L-Trp binding region and he hypothesizes that this space may be available for a natural cofactor or a regulator of the enzyme (30). Interestingly, several of the β-carboline inhibitors, such as **67**, **70** and **72**, demonstrated considerably greater potency on preincubation with IDO. This is suggestive of slow binding inhibition and may indicate these inhibitors need time to settle into the second binding pocket near the heme iron. One important liability of β-carboline derivatives is the reported neuroactivity of these structures as benzodiazepine receptor ligands (45-48). In fact, many previous IDO inhibitor studies were focused on developing treatments for neurological disorders (*e.g.*, excitotoxic brain lesions) where penetration of the central nervous system may have been necessary. However, an IDO inhibitor able to penetrate the central nervous system could cause problematic side effects in cancer therapy.

2. Miscellaneous structures

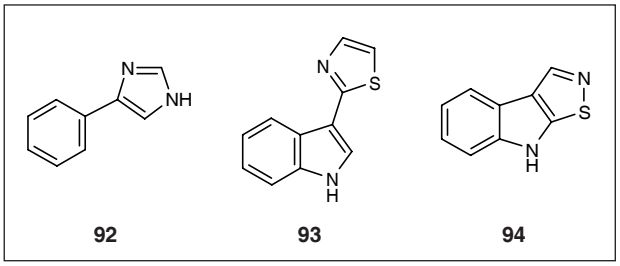
A small group of other compounds have been discovered to be noncompetitive inhibitors. Although limited in number, these structures provide some unique and potent structural leads. 4-Phenylimidazole **92** is believed to bind to the heme iron, similar to β-carboline (**65**) (30). It seems possible that brassilexin (**94**) may also bind to the heme iron through the sulfur of the isothiazole ring.

Although a selection of compounds have been investigated for IDO inhibition, submicromolar inhibition has not yet been achieved. A few unique structures have been discovered to have IDO-inhibitory activity, athough

Table VI: Other compounds demonstrating noncompetitive inhibition.

Compound	Structure	Inhibition data (%)[a]	Ref.
92	4-phenylimidazole	K_i = 4.4 μM	43
93	camalexin	21.3	44
94	brassilexin	K_i = 5.4 μM	44

[a] Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an *in vitro* competitive inhibition assay with 100 μM of inhibitor.



92 **93** **94**

the majority of the most active structures contain the indole core or resemble L-Trp. Clearly, one important goal in the development of IDO inhibition as a cancer therapy will be to discover more potent inhibitors, and it seems that a diversification of IDO inhibitor structures may be necessary to achieve this goal.

Therapeutic potential of IDO inhibitors

IDO suppresses activation of T-cell immunity

IDO is an extrahepatic enzyme that catalyzes the initial and rate-limiting step in the degradation of tryptophan along the kynurenine pathway that leads to the biosynthesis of NAD^+ (nicotinamide adenine dinucleotide) (26, 49). IDO does not, however, handle dietary catabolism of tryptophan, which is instead the role of the structurally unrelated liver enzyme tryptophan dioxygenase (TDO2). Moreover, NAD^+ levels in mammalian cells are predominantly maintained by salvage pathways. Thus, for many years the biological role of IDO remained unclear. Recently, however, it has been demonstrated that IDO modulates immune function by suppressing cytotoxic T-cell activation (reviewed in 50). The physiological relevance of IDO-mediated immunosuppression was confirmed in a seminal study which demonstrated that administration of the bioactive IDO inhibitor 1-MT (16) can elicit MHC-restricted, T-cell-mediated rejection of allogeneic mouse concepti (28, 51).

Genetic control of IDO by the tumor suppressor gene Bin1

Elevated tryptophan catabolism in cancer patients, first reported in the 1950s (52), was generally ascribed to be a tumoricidal effect of interferon gamma elevation

operating through IDO to starve the rapidly growing tumor cells of the essential amino acid tryptophan (53). However, the elucidation of IDO's toleragenic role has recently prompted the opposing hypothesis that elevated IDO can promote tumor cell immune escape by suppressing the activation of cytotoxic T-cells that could recognize and destroy them.

A key finding in support of this hypothesis has been the discovery of a regulatory link between IDO elevation in tumor cells and an established cancer suppression signaling pathway controlled by the adaptor protein Bin1 (39). Bin1 was originally identified through its ability to interact with and inhibit the oncogenic activity of the c-Myc oncoprotein (54, 55). Subsequent studies have indicated complex splice regulation of Bin1 protein isoforms in cells, which are linked to diverse cellular processes, and systemic disruption in homozygous *Bin1* knockout mice results in perinatal lethality associated with severe cardiomyopathy (56). Existing studies in human prostate and breast cancers support the candidacy of *Bin1* as a tumor suppressor or negative modifier gene. Loss or attenuation of *Bin1* expression occurs in > 50% of primary human breast tumors and in all breast tumor cell lines examined to date (57). The 2q14-21 region, where *Bin1* is located, is frequently deleted in breast cancers (58, 59), particularly in tumors that contain *BRCA1* mutations or have metastatic capacity (58-61). In prostate cancers, *Bin1* shows frequent loss of heterozygosity (LOH) and loss of expression, especially in advanced cases with metastatic capacity (62, 63). Studies utilizing *Bin1* knockout mouse-derived cell lines corroborate the hypothesis that *Bin1* has an antiprogression role in cancer. In particular, *Bin1* loss provides a dramatic cell-extrinsic benefit to *in vivo* tumor growth that is explained by IDO dysregulation (39).

Preclinical studies combining IDO inhibitors with breast cancer chemotherapy

Based on these pivotal studies linking *Bin1* loss to IDO upregulation and immune escape by tumors, critical proof-of-principle studies have been performed. These studies have led to the discovery of a novel therapeutic strategy whereby IDO inhibitors in combination with standard chemotherapeutic agents cooperatively produce dramatic regression of established tumors in preclinical studies (39). One prediction of the hypothesis framed in the previous section is that an IDO inhibitor might break immune tolerance and promote tumor regression. We employed the well-accepted mouse model of breast cancer, the MMTV-*Neu* "oncomouse" that develops mammary gland tumors closely resembling human ductal carcinoma *in situ* (DCIS), to investigate this idea. The possible antitumor effects of the well-established IDO inhibitor 1-MT were evaluated either alone or in combination with other agents. 1-MT treatment alone slowed tumor growth but did not reverse it, consistent with other recently published observations (64, 65). This finding suggests that single-agent IDO inhibitor-based immunotherapy has limited antitumor efficacy when applied to established tumors. In contrast, treatment of tumor-bearing MMTV-*Neu* mice with a combination of 1-MT + paclitaxel produced an average decrease of approximately 30% in tumor volume at the 2-week endpoint, while paclitaxel treatment by itself produced only tumor growth inhibition. Histological and immunohistochemical examination revealed evidence of increased cell death in tumors from mice treated with 1-MT + paclitaxel (39). Consistent with host immunity being critical for the therapeutic regression of tumors, immunodepletion of either $CD4^+$ T-cells (39) or $CD8^+$ T-cells (unpublished) abrogated the ability of 1-MT to cooperate with paclitaxel. Similar cooperativity was observed with some but not all chemotherapeutic agents tested (39).

In summary, IDO inhibition produces dramatic antitumor efficacy in the autochthonous MMTV-*Neu* tumor model when combined with certain cytotoxic chemotherapeutic agents. This finding is striking as it supports what has generally been viewed as a counterintuitive notion, that combining immunotherapy with chemotherapy can be used to effectively promote tumor regression. Immunotherapy and chemotherapy have generally been thought to work at cross purposes; however, the case for complementarity has been gaining as of late, based on experiments employing increasingly sophisticated models and tools to monitor the progress of antitumor immune responses (66).

While a useful tool for proo-of-principle studies, 1-MT is not an ideal compound for development as a clinical agent. 1-MT is a weak inhibitor, especially in cell-based assays (where the EC_{50} is > 200 μM), and has poor solubility characteristics. To address these issues, we have conducted enzyme- and cell-based screens of commercially available compounds and have identified a series of thiohydantoin derivatives of tryptophan that are pharmacologically superior to 1-MT. The most potent of these is the compound 3'-methylthiohydantointryptophan (termed MTH-trp), with an EC_{50} in the cell-based assay of 12 μM (39), being approximately 20-fold more potent than 1-MT.

A trial of MTH-trp in the MMTV-*Neu* autochthonous tumor model revealed that it has biological activity similar to or better than that of 1-MT. Over the 2-week course of treatment, MTH-trp alone promoted tumor growth delay but in combination with paclitaxel promoted tumor regression. MTH-trp combination therapy achieved an overall reduction in mean tumor volume over the 2-week treatment period of 58% (including one complete regression) as compared to 30% with 1-MT. As with 1-MT combination therapy, tumor regression was found to be associated with increased tumor cell death (39).

Therapeutic potential of IDO inhibitors based on market analysis

Cancer is the second leading cause of death in the U.S., with over 500,000 people dying each year. Of the

approximately 1.4 million new cases of cancer diagnosed in 2004, 563,700 will have died in 2004 (41% death rate). Of these deaths, 28% will have been from lung cancer, 10% from colon cancer, 7% from breast cancer and 5% from prostate cancer. These cancers are often referred to as "The Big Four" to represent the major targets to positively impact the cancer survival rate. Breast cancer is the most frequently diagnosed cancer in women. In 2002, approximately 200,000 new cases were diagnosed in the U.S. and over 1 million new cases worldwide. Treatment for breast cancer is correlated to the disease stage and patient hormone receptor status, with specific therapy decisions based on an individual patient's likelihood of response. Typical interventions for breast cancer range from surgical resection, for the treatment of localized stage 1 tumors, to combination treatment strategies that employ surgery, radiotherapy and multidrug therapy for patients with advanced breast cancer. Current treatment of metastatic disease, or stage IV disease, is intended to prolong the patient's life balanced against the impact of treatment on the patient's quality of life. In breast cancer, fewer than 15% of patients who develop metastatic disease survive for 5 years after diagnosis, irrespective of treatment.

The extraordinary size of the cancer market becomes apparent in Table VII, which shows sales (in millions) of chemical anticancer drugs in the U.S., with 2005 projected (as denoted by the asterisk).

The market for cancer chemotherapies in 2001 reached $4 billion in the U.S. and $10.8 billion worldwide, and is growing at an annual rate of 10%. Of this market, breast cancer therapies represented approximately $250 million annual sales in the U.S. The antitubulin segment, of which Taxol® (paclitaxel) is a member, is projected to be the fastest growing segment of the cancer therapies market. Taxol® sales reached $1.2 billion in 1998 and $1.7 billion in 2001. The expanded use of Taxol® (and the newer taxanes), as represented by the over 20% per year increase in sales since its introduction, reflects both its utility and the very limited alternatives available for the effective treatment of solid tumors. IDO inhibitors, which are likely to work most effectively as immunomodulatory adjuncts to conventional chemotherapeutics, represent particularly attractive candidates for clinical development in this context. As such, IDO inhibitors have tremendous potential to cooperatively leverage taxane-based breast cancer therapy, as well as other chemical therapies for a variety of cancer indications.

Table VII: U.S. market for cancer therapies.

Therapy	1995	2000	2005*
Cytotoxic drugs	$1,397	$1,863	$1,975
Antimetabolites	$265	$338	$355
Antitubulin agents	$580	$783	$846
Alkylating agents	$220	$281	$295
Others	$305	$378	$412

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Structure−Activity Study of Brassinin Derivatives as Indoleamine 2,3-Dioxygenase Inhibitors

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Received September 8, 2005

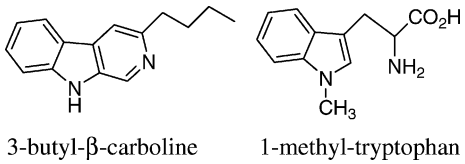
A screen of indole-based structures revealed the natural product brassinin to be a moderate inhibitor of indoleamine 2,3-dioxygenase (IDO), a new cancer immunosuppression target. A structure−activity study was undertaken to determine which elements of the brassinin structure could be modified to enhance potency. Three important discoveries have been made, which will impact future IDO inhibitor development: (i) The dithiocarbamate portion of the brassinin lead is a crucial moiety, which may be binding to the heme iron of IDO; (ii) an indole ring is not necessary for IDO inhibition; and (iii) substitution of the S-methyl group of brassinin with large aromatic groups provides inhibitors that are three times more potent in vitro than the most commonly used IDO inhibitor, 1-methyl-tryptophan.

Introduction

Understanding how tumors escape the host immune system is a rapidly developing area in the field of cancer research. Recently, the enzyme indoleamine 2,3-dioxygenase (IDO; EC 1.13.11.42) has been implicated in tumor immunosuppression.[1] Several reports identify IDO as playing a role in undermining a more vigorous immune response to tumor growth. Consequently, we have been focused on identifying novel, potent IDO inhibitors with the goal of developing a new therapeutic approach to cancer treatment. IDO inhibitors alone or in combination with other chemotherapeutics might provide another tool in the oncology armamentarium.

IDO is an extrahepatic enzyme that catalyzes the initial and rate-limiting step in the degradation of tryptophan (Trp) along the kynurenine pathway that leads to the biosynthesis of nicotinamide adenine dinucleotide.[2] IDO does not, however, handle dietary catabolism of Trp, which is instead the role of the structurally unrelated liver enzyme Trp dioxygenase (EC 1.13.11.11). IDO is a monomeric 45 kDa heme-containing oxidase that is active with the heme iron in the ferrous (Fe^{2+}) form. The ferric (Fe^{3+}) form of IDO is inactive, and substrate inhibition is believed to result from Trp binding to ferric IDO.[3] The primary catalytic cycle of IDO does not involve redox changes; nevertheless, IDO is prone to autoxidation; therefore, a reductant is necessary to reactivate the enzyme. In vivo, IDO purportedly relies on a flavin or tetrahydrobiopterin cofactor. In vitro, methylene blue and ascorbic acid are believed to substitute for the natural flavin or tetrahydrobiopterin cofactor.

Inhibition of IDO has previously been targeted for other therapies, most notably neurological disorders.[2b] Several metabolites of the kynurenine pathway are neurotoxic or are implicated in neurodegeneration, e.g., quinolinic acid; therefore, attention has focused on IDO. A recent review[4] summarizes the range of compounds that have been tested as IDO inhibitors. Strikingly, almost all IDO inhibitors, whether competitive or noncompetitive, retain the indole ring of the natural substrate. Currently, the most potent IDO inhibitor reported is 3-butyl-β-carboline (Figure 1), a noncompetitive inhibitor with a K_i = 3.3 μM.[5] However, the most commonly used IDO inhibitor is 1-methyl-Trp (Figure 1),[6] a commercially available compound that is a competitive inhibitor with a K_i = 34 μM.



3-butyl-β-carboline 1-methyl-tryptophan

Figure 1. IDO inhibitors.

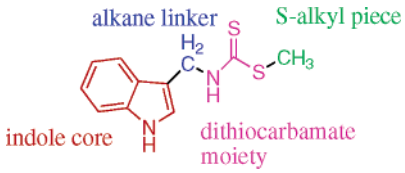


Figure 2. Brassinin (**1**) structure.

We undertook a screen of commercially available indole-based molecules to find novel IDO inhibitors. Interestingly, the natural product brassinin (**1**; Figure 2) was found to be a moderately active competitive inhibitor, K_i = 97.7 μM. Brassinin is a phytoalexin in cruciferous plants[7] and has demonstrated some antifungal[8] and anticancer activity.[9] We undertook a structure−activity relationship study of brassinin with the goal of obtaining a more potent IDO inhibitor. We divided the brassinin structure into four components: the indole core, the alkane linker, the dithiocarbamate moiety, and the S-alkyl piece. Analogues of brassinin that varied each of the four components were synthesized. The study determined the significance and flexibility of each of the four portions of the brassinin structure. The experimentation led to more potent inhibitors and several important developments in the field of IDO inhibition. Details of this study and our findings are reported herein.

Results and Discussion

Chemistry. Brassinin dithiocarbamate analogues were synthesized by adding an amine to carbon disulfide at 0 °C, stirring for 1 h, and then adding an alkyl halide. Modification of the indole core or alkane linker occurred by adding different amines. Many amines were commercially available, although several

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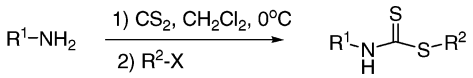
10.1021/jm0508888 CCC: $33.50
Published on Web 12/17/2005

Scheme 1. Dithiocarbamate Synthesis



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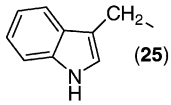
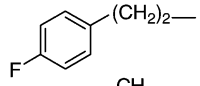
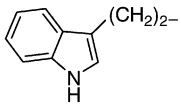
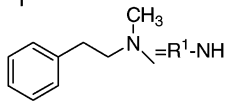
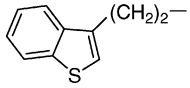
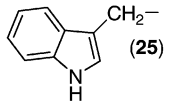
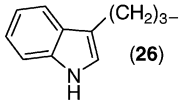
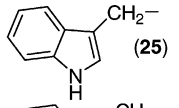
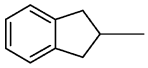
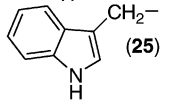
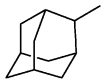
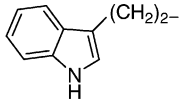
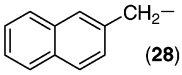
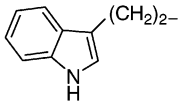
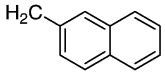
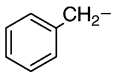
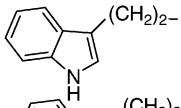
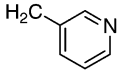
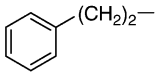
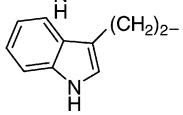
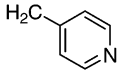
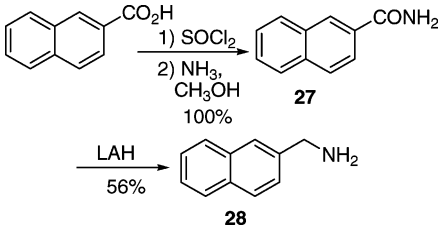
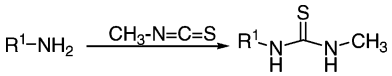
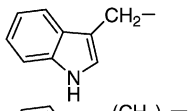
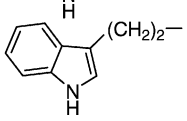
1		CH_3	43	**10**		CH_3	89
2		CH_3	87	**11**		CH_3	85
3		CH_3	22	**12**		$H_2C{=}CH_2$	52
4		CH_3	61	**13**		H_2C–Ph	50
5		CH_3	74	**14**		$-(CH_2)_5CH_3$	57
6		CH_3	98	**15**		H_2C–Ph	86
7		CH_3	54	**16**			59
8		CH_3	74	**17**			40
9		CH_3	85	**18**			50

Scheme 2



Scheme 3. Thiourea Synthesis

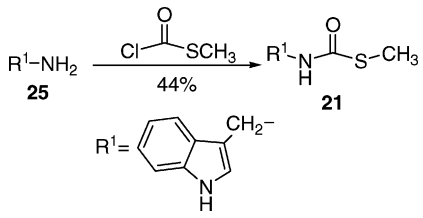


	R_1	Yield (%)
19		54
20		92

required synthesis. The indole-3-methanamine **25** of brassinin **1** was prepared through the reductive amination of indole-3-carboxaldehyde. Although there are several different reductive amination procedures reported in the literature,[9,10] we found Mehta's procedure[9a] to be the most effective. Homotryptamine **26**, the amine reagent for **4**, was synthesized in three steps from indole-3-propanoic acid following literature precedent.[11] 2-Aminomethyl-naphthalene **28** was also synthesized in three steps from 2-naphthoic acid (Scheme 2). Modifications of the S-alkyl piece occurred by substituting various alkyl halides for iodomethane, e.g., **12**−**18** (Scheme 1).

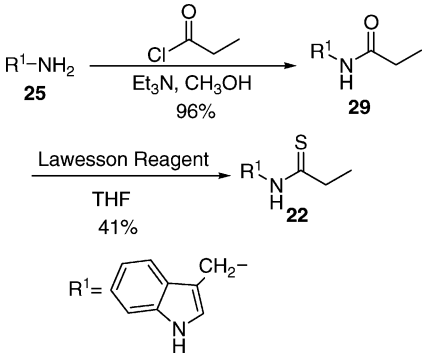
Modifications to the dithiocarbamate moiety included thioureas (**19** and **20**; Scheme 3), S-alkyl thiocarbamates (**21**; Scheme 4), thioamides (**22**; Scheme 5), and thiazoles (**23** and **24**; Scheme 6). The thioureas were synthesized by reacting amines with methyl isothiocyanate (Scheme 3).[35] The S-alkyl thiocarbamate **21**, a phytoalexin called brassitin, came from the

Scheme 4. Brassitin Synthesis



reaction of S-alkyl thiochloroformate with **25** (Scheme 4). The thioamide **22** was synthesized by reaction of **25** with an acid chloride and then treatment with Lawesson's reagent (Scheme 5). Thiazoles were synthesized by reaction of thioformamide or thioacetamide with α-bromoketones **31** (Scheme 6). The

Scheme 5. Thioamide Synthesis



Scheme 6. Thiazole Synthesis

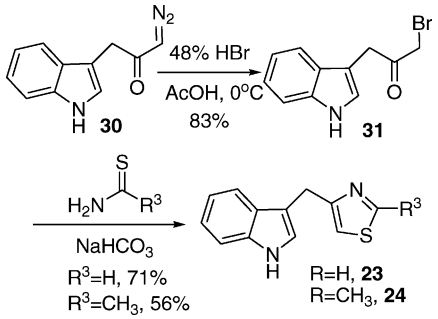


Table 1. IDO Inhibition Data

compound	K_i (μM)	compound	K_i (μM)	compound	K_i (μM)
1	97.7	**9**	62.36	**17**	28.38
2	82.54	**10**	149.35	**18**	20.48
3	40.95	**11**	1267	**19**	NI[a]
4	33.97	**12**	36.95	**20**	342.3
5	42.06	**13**	13.22	**21**	NI
6	179.6	**14**	363.6	**22**	202
7	47.57	**15**	17.15	**23**	1292
8	72.41	**16**	11.55	**24**	328.7

[a] NI, no inhibition detected.

α-bromoketones **31** were generated from the corresponding α-diazoketone derivative **30**,[12] which was synthesized in three steps from indole-3-acetic acid following a literature procedure.[13]

Enzyme Studies. Brassinin analogues were analyzed for inhibition of extracted and purified recombinant human IDO produced in bacteria. The assay was conducted according to a literature protocol,[14] with ascorbic acid and methylene blue serving the role of reductant.[15] Catalase was added to prevent IDO decomposition from peroxide side products.[16] The enzyme assay was monitored for formation of N-formylkynurenine by hydrolyzing the formyl group and spectrophotometrically analyzing for the conjugated imine generated from kynurenine and 4-(dimethylamino)benzaldehyde. In all cases where inhibition was seen, the brassinin analogues demonstrated competitive inhibition. The inhibitory constants shown are an average of two or three trials. The array of analogues tested allowed for an evaluation of the four components of the brassinin structure and resulted in some important discoveries (Table 1).

Variation of the Indole Core. One of the most suprising discoveries was the range of groups that could be substituted for indole and still retain some IDO inhibitory activity. Indeed, not only were flat aromatic structures (e.g., **5** and **7**−**10**) effective substitutes, but the adamantyl structure **6** could also bind in the substrate pocket, based on the competitive inhibition witnessed for all of these analogues. Although IDO is relatively promiscuous, there are still very few reports of substrates or inhibitors that lack the indole core.[4] The current demonstration of inhibition with benzene and cycloalkyl-based structures expands the range of structures that behave as IDO inhibitors. Furthermore, benzene aromatic structures are more easily derivatized with available synthetic methods than indole compounds. Indole derivatives can also be a liability given the neuroactivity of some indole-containing compounds, e.g., serotonin and related indolealkylamines.

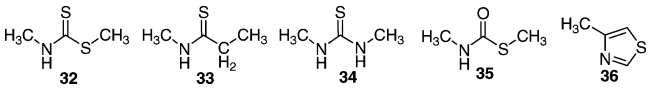


Figure 3. Dithiocarbamate analogues.

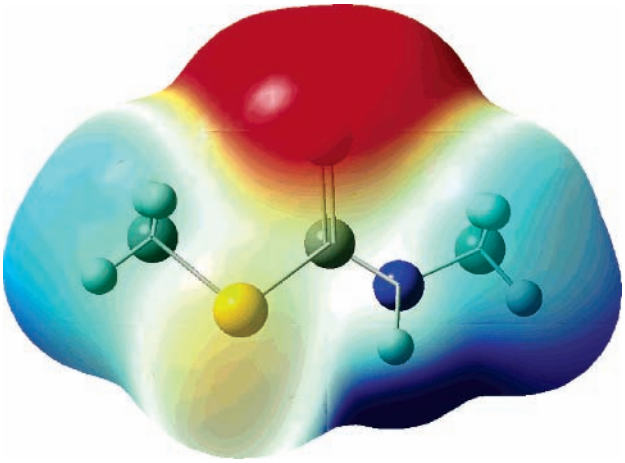


Figure 4. Molecular ESP mapped onto electron distribution for **32**.

Variation of the Alkane Linker. Linker variation was possible, and in the brassinin series, it was found that the longer linker led to more potent compounds, cf. **1** vs **2** vs **4**. However, analogues that modified two brassinin components did not replicate this trend (cf. **13** vs **15**). Taken together, the results with the alkane linker modifications and the indole core changes indicate that the IDO active site is rather accommodating.

Variation of the Dithiocarbamate. The most interesting results came from isosteric modifications of the dithiocarbamate. The transformation of brassinin's dithiocarbamate moiety to a thiourea (**19** and **20**), thiocarbamate (**21**), thioamide (**22**), or thiazole (**23** and **24**) led to weaker or no inhibition. Notably, the S-methyl-thiocarbamate analogue **21** (brassitin),[29] suffered a complete loss in inhibitory activity with the substitution of a carbonyl for the thiocarbonyl group. Given the recognized metal-coordinating properties of dithiocarbamates,[17,18] it is likely that the dithiocarbamate moiety is chelating to the heme iron at the active site of IDO.[19] In fact, pyrrolidine dithiocarbamate reportedly inhibits IDO,[17a] besides being a well-known antioxidant and NF-κB inhibitor.[20]

Computational Experiments to Explore the Electronic Nature of Dithiocarbamate. If the dithiocarbamate moiety is binding to the heme iron, then the electronic nature or charge of the group should be important to achieve optimum binding. We performed several computational experiments to understand the electronic nature of the dithiocarbamate sulfur vs the sulfur/oxygen in the isosteric analogues. To reduce the computational time, most of the experiments involved simplified analogues **32**−**36** (Figure 3), which lacked the indole ring. Figure 4 shows the molecular electrostatic potential (ESP) mapped onto the electron distribution of **32**, the dithiocarbamate analogue. Clearly, the sulfur of **32** projects the greatest electron density and therefore is the richest and most available Lewis basic site for iron binding.

Table 2. Charge Data for Dithiocarbamate Analogues

compound	charge ESP	charge NBO	K_i of related analogue (μM) (compound no.)
32	−0.466	−0.277	97.7 (**1**)
33	−0.518	−0.318	202 (**22**)
34	−0.550	−0.390	NI (**19**)
35	−0.661	−0.711	NI (**21**)
36	−0.133	0.290	1292 (**23**)

In Table 2, the ESP and natural bond order (NBO) charge values were derived for **32**−**36**. Elimination of the indole group allowed for more rapid computational experiments and did not affect the trend witnessed.[21] For compounds **32**−**35**, a clear trend can be seen for both ESP and NBO charge calculations: the smaller the charge on sulfur/oxygen, the better the inhibition. Compound **36** breaks the trend; however, the conformationally rigid thiazole ring may prevent an effective interaction between the sulfur of the thiazole and the heme iron. The trend witnessed with compounds **32**−**35** may seem counterintuitive, i.e., a more electron rich sulfur/oxygen should be a better electron donor to the Lewis acid heme iron. Nevertheless, optimum inhibition may arise from a softer Lewis base coordinating with the softer active ferrous form of the enzyme.[22] Future inhibitor design will be guided by the insights from these computational experiments.

Variation of the S-Alkyl Piece. The greatest increases in potency were realized in modifications of the S-alkyl group. Although alkyl groups that were longer than the methyl in brassinin were less active, S-allyl brassinin **12** was two times more potent than brassinin and the benzyl analogue **13** was almost one order of magnitude more potent. Moreover, the tryptamine/naphthyl analogue **16** was as potent as **13**; pyridyl analogues **17** and **18** also demonstrated modest inhibition. Because all these compounds behaved as competitive inhibitors, these analogues reveal a large additional pocket in the IDO active site capable of accommodating flat, aromatic groups.

Sono has reported that β-carboline, a noncompetitive inhibitor, binds to the heme iron at the active site but not in the same space as the substrate.[23] Moreover, the β-carboline reportedly acts as a nitrogen donor ligand and competes with O_2 for binding to the heme iron. It is possible that the large aromatic S-alkyl pieces are binding in the same pocket that accommodates the tricyclic aromatic β-carboline structure. Nevertheless, the pyridyl analogues **17** and **18** failed to demonstrate stronger inhibition despite their similarity to the pyridyl ring of β-carboline.

Conclusion

A systematic study of the IDO inhibitory activity of brassinin has been undertaken, and three important discoveries have been made. Contrary to most previously reported IDO inhibitors, an indole ring was not necessary for inhibitory activity with the dithiocarbamate analogues of brassinin. Although indole-containing derivatives were still the most active inhibitors (i.e., **13** and **16**), the inhibitory activity retained by analogues, such as **5** and **7**−**9**, create new opportunities to further inhibitor development. Importantly, new analogues might be possible that avoid the pharmacological liabilities of the indole ring and leverage the wealth of chemical methods for benzene substitution. The dithiocarbamate moiety is an optimum group for IDO inhibition and probably chelates to the active site iron. Large unsaturated groups on the dithiocarbamate sulfur can be accommodated in the active site and lead to more potent inhibitors of IDO. Although only small increases in potency were achieved through the structure−activity study, the new inhibitors (i.e., **13** and **16**) are three times more potent than the most commonly used IDO inhibitor. In addition, the structure−activity relationship discoveries should greatly advance the search for more potent IDO inhibitors, an exciting new cancer target.

Experimental Section

Chemistry. All reactants and reagents were commercially available and were used without further purification unless otherwise indicated. Anhydrous THF was obtained by distillation from benzophenone-sodium under argon immediately before use. Anhydrous CH_2Cl_2 and Et_3N were obtained by distillation from calcium hydride under argon. Methanol was dried over Mg and distilled under argon. A saturated solution of HCl in CH_3OH was made by bubbling HCl through a drying tube, filled with $CaCl_2$, into a cooled flask of anhydrous CH_3OH under a stream of argon. A saturated solution of NH_3 in CH_3OH was made by bubbling anhydrous NH_3 into an Erlenmyer flask with a predetermined volume of CH_3OH. Concentrated refers to the removal of solvent with a rotary evaporator at normal water aspirator pressure followed by further evacuation with a two-stage mechanical pump unless otherwise indicated. Yields refer to chromatographically and spectroscopically pure (>95%) compounds, except as otherwise indicated. All new compounds were determined to be >95% pure by nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC), and/or gas chromatography (GC) as indicated (see Supporting Information). Melting points were determined using an open capillary and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively. Chemical shifts are reported in δ values (ppm) relative to an internal reference (0.05% v/v) of tetramethylsilane (TMS) for ^{1}H NMR and the solvent peak in ^{13}C NMR, except where noted. Peak splitting patterns in the NMR are reported as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad. Rotamer peaks (about 1/4 intensity) were seen for all dithiocarbamate structures.[24] Normal phase HPLC (NP-HPLC) analysis was performed with UV detection at 254 nm and a 5 μm silica gel column (250 mm × 4.6 mm), eluted with 90:10 *n*-hexane:IPA (or gradient) at 1 mL/min. Reverse phase HPLC (RP-HPLC) analysis was performed with UV detection at 254 nm and a C_{18} column (300 mm × 3.9 mm), eluted with a gradient of H_2O + 0.1% TFA and CH_3CN + 0.1% TFA at 1 mL/min., unless otherwise indicated. GC analyses were performed with an EI-MS detector fitted with a 30 m × 0.25 mm column filled with cross-linked 5% PH ME siloxane (0.25 μm film thickness); gas pressure 7.63 psi He. IR data were obtained with an FT-IR spectrometer. Thin-layer chromatography (TLC) was performed using silica gel 60 A precoated glass-backed plates (0.25 mm thickness) with fluorescent indicators, which were scored and cut. Developed TLC plates were visualized with UV light (254 nm), iodine, or $KMnO_4$. Flash column chromatography was conducted with the indicated solvent system using normal phase silica gel 60 A, 230−400 mesh. All reactions were carried out under an inert atmosphere of argon or nitrogen unless otherwise indicated.

Indole-3-methanamine (25). Indole-3-carboxaldehyde (189 mg, 1.3 mmol) and NH_4OH·HCl (113 mg, 1.63 mmol) were dissolved in a Parr flask with 15 mL of MeOH, which was previously saturated with anhydrous ammonia. The flask was stoppered and placed on a Parr shaker for 5 h. To the resulting solution was added 200 mg of Raney nickel (50% slurry in H_2O), and the flask was pressurized to 60 psi with H_2 and allowed to shake overnight. The next day, the resulting mixture was filtered through Celite and volatiles were removed to yield 190 mg (100% yield) of a yellow solid. The product was unstable, so it was used immediately in subsequent reactions without further purification. ^{1}H NMR ($CDCl_3$/CD_3OD): δ 8.04 (br s, 1H, N*H*), 7.67 (d, 1H, Ar*H*, J = 7.8), 7.39 (d, 1H, Ar*H*, J = 7.1), 7.16 (3H, Ar*H*), 4.07 (d, 2H, ArCH_2, J = 7.4 Hz).

General Method for the Synthesis of Dithiocarbamates. The amine (1.0 equiv) was dissolved in pyridine (2−3 mL), and the solution was cooled to 0 °C. Triethylamine (1.0−1.1 equiv) and carbon disulfide (1.1 equiv) were added, and the solution was stirred

at 0 °C. After 30 min, iodomethane (1.0−1.2 equiv) was added and the reaction was allowed to slowly warm to room temperature overnight. The reaction was poured into 1 M H_2SO_4 and extracted with EtOAc (3×). The organic layer was washed with brine, dried with Na_2SO_4, and filtered. Concentration afforded a crude product that was chromatographed as described.

Brassinin (1). Brassinin was formed from **25** according to the general method. The crude yellow solid was chromatographed on silica with EtOAc/hexanes (1:3), and the resulting yellow solid was further purified by recrystallization from CH_2Cl_2/hexanes to yield rose-colored crystals (43% yield). ^{1}H and ^{13}C NMR spectra, IR spectrum, and mp matched a previous report for **1**.[25]

N-[2-(Indol-3-yl)ethyl]-S-methyl-dithiocarbamate (2).[26] The general method was used with tryptamine and CH_2Cl_2 as the solvent. The crude product was chromatographed with CH_2Cl_2/hexanes (2:1) to afford a waxy yellow-white solid (87% yield); mp = 59−64 °C. ^{1}H NMR ($CDCl_3$): δ 8.05 (br s, 1H, N*H*), 7.61 (d, 1H, Ar*H*, J = 7.8 Hz), 7.36 (d, 1H, Ar*H*, J = 8.0 Hz), 7.22 (dt, 1H, Ar*H*, J = 8, 1 Hz), 7.14 (dt, 1H, Ar*H*, J = 8, 1 Hz), 7.01 (br s, 1H, N*H*), 4.05 (q, 2H, C*H*$_2$NH, J = 12.4, 6.6 Hz), 3.11 (t, 2H, ArC*H*$_2$, J = 6.7 Hz), 2.56 (s, 3H, C*H*$_3$) and signals due to a minor rotamer (ca. 29%) at 3.73 (m), 2.68 (s). ^{13}C NMR ($CDCl_3$): δ 198.8, 136.4, 127.1, 122.4, 122.1, 119.7, 118.7, 112.3, 111.3, 47.2, 23.9, 18.0, and signals due to a minor rotamer at 201.6, 126.8, 118.4, 46.1, 24.6, 18.9. GC: t_R = 15.00 min. EI-MS m/z (%): 202 (27, M^+-SCH_3), 143 (4), 130 (100). NP-HPLC t_R = 5.9 min.

N-[2-(Benzo[*b*]thiophen-3-yl)ethyl]-S-methyl-dithiocarbamate (3). The general method was used with 2-(benzo[*b*]thiophen-3-yl)ethanamine and CH_2Cl_2 as the solvents. The crude product was chromatographed with EtOAc/hexanes (1:9) to yield a light amber oil, which slowly crystallized (22% yield); mp = 81−84 °C. ^{1}H NMR ($CDCl_3$): δ 7.90 (m, 1H, Ar*H*), 7.79 (m, 1H, Ar*H*), 7.43 (m, 3H, Ar*H*), 7.02 (br s, 1H, N*H*), 5.18 (d, 2H, ArCH$_2$C*H*$_2$, J = 4.17 Hz), 2.63 (m, 5H, SC*H*$_3$ overlapping with ArC*H*$_2$) and a signal due to a minor rotamer (ca. 17%) at 4.91 (m). ^{13}C NMR ($CDCl_3$): δ 199.2, 140.6, 137.7, 130.7, 125.9, 124.9, 124.7, 123.1, 121.7, 45.1, 18.3, 14.2 and signals due to minor rotamer peaks at 60.4, 21.0. IR (KBr) ν_{max} cm^{-1}: 3336, 3229, 3079, 2995, 2916, 1499, 1379, 1302, 1075, 926. NP-HPLC t_R = 4.8 min. RP-HPLC t_R = 12.1 min.

N-[3-(Indol-3-yl)propyl]-S-methyl-dithiocarbamate (4). The general method was used with 3-(indol-3-yl)-propan-1-amine, 2 equiv of Et_3N, and MeOH as the solvents. After the reaction was complete, the volatiles were removed and the residue was dissolved in EtOAc (60 mL). The solution was washed with 0.5 M HCl (2 × 30 mL), H_2O (20 mL), and brine (20 mL). The organic solution was dried with Na_2SO_4, filtered, and concentrated. The crude product was chromatographed with EtOAc/hexanes (1:3) to yield an off-white oil, which crystallized overnight (61% yield); mp = 54−56 °C. ^{1}H NMR ($CDCl_3$): δ 8.04 (br s, 1H, N*H*), 7.60 (d, 1H, Ar*H*, J = 7.6), 7.35 (d, 1H, Ar*H*, J = 8.0), 7.20 (m, 1H, Ar*H*), 7.12 (m, 1H, Ar*H*), 7.04 (m, 1H, Ar*H*), 6.91 (br s, 1H, N*H*), 3.82 (q, 2H, ArCH$_2$CH$_2$C*H*$_2$, J = 7.0 Hz), 2.86 (t, 2H, ArC*H*$_2$CH$_2$ J = 7.2 Hz), 2.52 (s, 3H, SC*H*$_3$), 2.1 (m, 2H, ArCH$_2$C*H*$_2$) and signals due to a minor rotamer (ca. 30%) 3.50 (q, J = 6.3 Hz), 2.68 (s). ^{13}C NMR ($CDCl_3$): δ 198.7, 136.4, 127.1, 122.2, 121.6, 119.4, 118.7, 115.1, 111.3, 47.2, 28.3, 22.7, 18.0 and signals due to a minor rotamer at 46.0, 28.9, 18.7. IR (KBr) ν_{max} cm^{-1}: 3410, 3321, 2919, 1888, 1504, 1337, 1094. EI-MS: m/z (%) 216 (57, M^+-SCH_3), 183 (5), 156 (10), 131 (23). NP-HPLC t_R = 12.3 min. RP-HPLC t_R = 11.9 min.

N-(Indan-2-yl)-S-methyl-dithiocarbamate (5). The general method was used with 2-aminoindan HCl. The crude product in EtOAc was decolorized with charcoal and filtered through Celite, and the volatiles were removed to yield a clear oil. The oil was chromatographed with EtOAc/hexanes (1:9) to yield an off-white solid (74% yield); mp = 106−108 °C. ^{1}H NMR ($CDCl_3$): δ 7.23 (4H, Ar*H*), 7.10 (br s, 1H, N*H*), 5.31 (m, 1H, CH$_2$C*H*CH$_2$), 3.44 (m, 2H, C*H*CHC*H*), 2.98 (dd, 2H, C*H*CHC*H*, J = 16.5 Hz, 3.7 Hz), 2.62 (s, 3H, SC*H*$_3$) and signals due to a minor rotamer (ca. 38%) 4.78 (m), 2.70 (s). ^{13}C NMR ($CDCl_3$): δ 198.6, 140.5, 127.0, 124.9, 57.9, 39.4, 18.2 and signals due to a minor rotamer at 57.1, 39.8, 18.5. IR (KBr) ν_{max} cm^{-1}: 3226, 2948, 2916, 2088, 1483, 1371, 1337, 1070. NP-HPLC t_R = 4.5 min. RP-HPLC t_R = 12.2 min.

N-(Adamant-2-yl)-S-methyl-dithiocarbamate (6). The general method was used with 2-adamantylamine HCl and 2 equiv of Et_3N to afford a white solid (98% yield); mp 128−129 °C. ^{1}H NMR ($CDCl_3$): δ 7.25 (br s, 1H, N*H*), 4.65 (t, 1H, C*H*NH, J = 3.6 Hz), 2.63 (s, 3H, SC*H*$_3$), 2.13 (m, 2H, C*H*$_2$), 1.73 (m, 12H, C*H*$_2$) and signals due to a minor rotamer (ca. 36%) 4.08 (m), 2.68 (s). ^{13}C NMR ($CDCl_3$): δ 197.8, 97.5, 61.1, 37.3, 32.7, 31.4, 27.4, 18.5 and signals due to a minor rotamer at 37.8, 32.0, 27.3, 19.3. IR (KBr) ν_{max} cm^{-1}: 3351, 2918, 2852, 1497, 1384, 1117, 942. NP-HPLC t_R = 4.1 min. RP-HPLC t_R = 13.2 min.

N-[(Naphth-2-yl)methyl]-S-methyl-dithiocarbamate (7). The general method was used with **28** and 2 equiv of Et_3N and MeOH as the solvents. The crude product was chromatographed on silica with EtOAc/hexanes (15:85) to yield a yellow solid (54% yield); mp 70−72 °C. ^{1}H NMR, ^{13}C NMR, and IR spectra matched a previous report for **7**.[27]

N-Benzyl-S-methyl-dithiocarbamate (8). The general method was used with benzylamine, and the crude product was chromatographed with EtOAc/hexanes (1:10) to yield an off-white oil (74% yield). ^{1}H NMR, ^{13}C NMR, and IR spectra matched a previous report for **8**.[28]

N-Phenethyl-S-methyl-dithiocarbamate (9). The general method was used with phenethylamine, and the crude product was chromatographed with EtOAc/hexanes (1:10) to yield an off-white solid (85% yield); mp 50−51 °C. ^{1}H NMR ($CDCl_3$): δ 7.28 (5 overlapping H, Ar*H*), 6.91 (br s, 1H, *NH*$_2$), 4.02 (t, 2H, ArCH$_2$C*H*$_2$, J = 6.9), 2.98 (t, 2H, ArC*H*$_2$CH$_2$, J = 7.0), 2.61 (s, 3H, SC*H*$_3$) and signals due to a minor rotamer (ca. 24%) at 3.71 (m), 2.69 (s). ^{13}C NMR ($CDCl_3$): δ 199.1, 138.2, 128.8, 128.7, 126.8, 48.0, 34.2, 18.1 and signals due to a minor rotamer at 47.3, 34.9, 18.5. IR (KBr) ν_{max} cm^{-1}: 3340, 3240, 3026, 2918, 1946, 1496, 1337, 1095. NP-HPLC t_R = 4.6 min. RP-HPLC t_R = 11.5 min.

N-4-Fluorophenethyl-S-methyl-dithiocarbamate (10). The general method was used with 4-fluorophenethylamine, and the solvent was CH_2Cl_2. The volatiles were removed, and the residue was dissolved in EtOAc. The organic layer was washed with 1 M H_2SO_4 (40 mL), H_2O (40 mL), and brine (30 mL). The resulting organic solution was dried with Na_2SO_4 and filtered. The volatiles were removed to yield a beige solid, which was chromatographed with EtOAc/hexanes (8/92) to yield a white solid (89% yield); mp 59−60 °C. ^{1}H NMR ($CDCl_3$): δ 7.18 (m, 2H, Ar*H*), 7.01 (m, 2H, Ar*H*), 3.96 (q, 2H, ArCH$_2$C*H*$_2$, J = 7.0 Hz), 2.96 (t, 2H, ArC*H*$_2$, J = 7.1 Hz), 2.62 (s, 3H, SC*H*$_3$) and signals due to a minor rotamer (ca. 24%) at 3.69 (q, J = 6.6 Hz), 2.68 (s). ^{13}C NMR ($CDCl_3$): δ 199.4, 161.8 (d, J = 243 Hz), 133.9, 130.2, 115.8, 48.0, 33.5, 18.1 and signals due to a minor rotamer at 130.1, 47.2, 30.9, 18.5. IR (KBr) ν_{max} cm^{-1}: 3250, 3002, 2921, 1886, 1506, 1385, 1222, 940.8. NP-HPLC t_R = 5.1 min. RP-HPLC t_R = 11.6 min.

N,S-Dimethyl-N-phenethyldithiocarbamate (11). The general method was used with N-methylphenthylamine, and the solvent was CH_2Cl_2. The crude product was chromatographed with EtOAc/hexanes (1/19) to yield a white oil (85% yield). ^{1}H NMR ($CDCl_3$): δ 7.29 (m, 5H, Ar*H*), 4.25 (t, 2H, ArCH$_2$C*H*$_2$, J = 6.9 Hz), 3.20 (s, 3H, NC*H*$_3$), 3.01 (q, 2H, ArC*H*$_2$, J = 6.8 Hz), 2.66 (s, 3H SC*H*$_3$) and signals due to a minor rotamer (ca. 42%) at 3.89 (m), 3.47 (s). ^{13}C NMR ($CDCl_3$): δ 198.6, 138.9, 138.1, 129.3, 129.2, 129.1, 127.0, 59.5, 40.9, 32.9, 20.7 and signals due to a minor rotamer at 56.6, 44.6, 34.0. IR (KBr) ν_{max} cm^{-1}: 3025, 2917, 1949, 1808, 1485, 1386, 1292, 1185, 1100, 992.5. NP-HPLC t_R = 4.2 min. RP-HPLC t_R = 12.7 min.

S-Allyl-brassinin (12). The general method was used with **25**, but allyl bromide was substituted for iodomethane. The crude product was purified by chromatography on silica with EtOAc/hexanes (3/7) to afford an orange oil (52% yield). ^{1}H NMR ($CDCl_3$): δ 8.17 (br s, 1H, N*H*), 7.64 (d, 1H, Ar*H*, J = 7.8 Hz), 7.43 (d, 1H, Ar*H*, J = 8.1 Hz), 7.21 (3H, Ar*H*), 7.03 (br s, 1H, N*H*), 5.93 (m, 1H, SCH$_2$C*H*═CH$_2$), 5.22 (m, 2H, SCH$_2$CH═C*H*$_2$),

5.05 (d, 2H, ArC*H*$_2$, J = 4.4 Hz), 3.92 (d, 2H, SCH_2CH=CH_2, J = 7.7 Hz) and signals due to a minor rotamer (ca. 16%) at 4.69 (m), 4.09 (d, J = 7 Hz). ^{13}C NMR ($CDCl_3$): δ 196.3, 136.2, 132.7, 126.4, 122.7, 120.2, 118.6, 118.5, 111.5, 110.3, 43.1, 38.3 and signals due to a minor rotamer at 41.0, 39.5. IR (KBr) ν_{max} cm^{-1}: 3402, 2915, 1852, 1635, 1377, 1063. NP-HPLC t_R = 6.7 min. RP-HPLC t_R = 11.6 min.

S-Benzyl-brassinin (13). The general method was used with **25**, but benzyl bromide was substituted for iodomethane and CH_2Cl_2 was used as the solvent. The crude product was chromatographed on silica EtOAc/hexanes (3/7) to yield a translucent, yellow oil, which slowly solidified. Recrystallization from CH_2Cl_2/hexanes yielded a bright yellow solid (50% yield); mp 101−102 °C. ^{1}H NMR ($CDCl_3$): δ 8.22 (br s, 1H, N*H*), 7.62 (d, 1H, Ar*H*, J = 7.9 Hz), 7.28 (9H, Ar*H* + Ph*H*), 6.98 (br s, 1H, N*H*), 5.11 (d, 2H, ArCH_2, J = 3.9 Hz), 4.55 (s, 2H, CH_2Ph) and signals due to a minor rotamer (ca. 19%) at 4.77 (d, J = 4.5 Hz), 4.67 (s). ^{13}C NMR ($CDCl_3$): δ 196.4, 136.6, 136.3, 129.0, 128.6, 127.5, 126.5, 124.0, 122.8, 120.3, 118.7, 111.4, 110.7, 43.2, 39.9. IR (KBr) ν_{max} cm^{-1}: 3417, 3334, 3058, 1890, 1494, 1455, 1067. NP-HPLC t_R = 6.8 min. RP-HPLC t_R = 12.5 min.

S-Hexyl-brassinin (14). The general method was used with **25**, but 1-iodohexane was substituted for iodomethane. The crude product was chromatographed on silica with EtOAc/hexanes (3/7) to yield a golden oil (57% yield). ^{1}H NMR ($CDCl_3$): δ 8.18 (br s, 1H, N*H*), 7.65 (d, 1H, Ar*H*, J = 7.8 Hz), 7.43 (d, 1H, Ar*H*, 8.1 Hz), 7.22 (3H, Ar*H*), 6.99 (br s, 1H, N*H*), 5.06 (d, 2H, ArCH_2, J = 4.4 Hz), 3.26 (t, 2H, SCH_2, J = 7.5 Hz), 1.70 (m, 2H, SCH$_2$CH_2-CH$_2$CH$_2$CH$_2$CH$_3$), 1.39 (6H, SCH$_2$CH$_2$CH_2CH_2CH_2CH$_3$), 0.88 (t, 3H, CH_3, J = 7.5 Hz) and signals due to a minor rotamer (ca. 19%) at 4.79 (d, J = 4.8 Hz), 3.39 (t, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$): δ 197.6, 136.2, 126.4, 124.0, 122.6, 120.1, 118.6, 111.5, 110.5, 43.0, 35.4, 29.0, 28.5, 22.5, 14.0 and signals due to a minor rotamer at 42.0, 36.5. IR (KBr) ν_{max} cm^{-1}: 3409, 3328, 2955, 2927, 2855, 1620, 1494, 1456, 1379, 1094. NP-HPLC t_R = 6.0 min. RP-HPLC t_R = 13.8 min.

N-[2-(Indol-3-yl)ethyl]-S-benzyl-dithiocarbamate (15). The general method was used with tryptamine as the amine and CH_2Cl_2 as the solvent. Benzyl bromide was used as the alkylating agent in place of iodomethane. The crude product was chromatographed with EtOAc/hexanes (1:4) to yield white crystals (86% yield). Further purification was accomplished by recrystallization in EtOAc/hexanes to afford a 73% yield; mp = 79−81 °C. ^{1}H NMR ($CDCl_3$): δ 8.02 (br s, 1 H, N*H*), 7.58 (m, 1 H, Ar*H*), 7.37 (m, 6 H, Ar*H*), 7.32−7.18 (m, 1 H, Ar*H*), 7.13 (t, 1 H, Ar*H*, J = 9.0 Hz), 6.99 (m, 2 H, Ar*H*), 4.48 (s, 2 H, SCH_2), 4.05 (q, J = 6.0 Hz, 1 H, ArCH$_2$CH_2), 3.09 (m, 2 H, ArCH_2) and signals due to a minor rotamer (ca. 24%) at 4.59 (s), 3.74 (q, J = 6.0 Hz). ^{13}C NMR ($CDCl_3$): δ 197.2, 136.5, 136.3, 129.3, 128.9, 128.6, 127.6, 127.4, 127.1, 122.4, 122.1, 119.7, 118.7, 112.2, 111.3, 47.2, 39.8, 24.6, 23.9, and signals due to a minor rotamer at 135.7, 127.6, 118.4, 41.0, 24.6. IR (KBr) ν_{max} cm^{-1}: 3394, 3179, 1618, 1503, 1455, 1332, 1095, 936. EI-MS: *m*/*z* (%) 130 (100), 202 (37). GC t_R = 14.8 min. NP-HPLC t_R = 7.6 min. RP-HPLC t_R = 12.9 min. Anal. calcd for $C_{18}H_{18}N_2S_2$: C, 66.22; H, 5.56; N, 8.58; S, 19.64. Found: C, 66.19; H, 5.43; N, 8.42; S, 19.87.

N-[2-(Indol-3-yl)ethyl]-S-[(naphth-2-yl)methyl]dithiocarbamate (16). The general method was used with tryptamine as the amine and CH_2Cl_2 as the solvent. 2-(Bromomethyl)naphthalene was used as the alkylating agent in place of iodomethane. The crude product was chromatographed with EtOAc/hexanes (1:4) to afford the pure product (59% yield). Further purification was accomplished by recrystallization in EtOAc/hexanes to afford white crystals (29% yield); mp = 158−160 °C. ^{1}H NMR ($CDCl_3$): δ 8.05 (br s, 1 H, N*H*), 7.90 (m, 1 H, Ar*H*), 7.79 (t, J = 9.4 Hz, 4 H, Ar*H*), 7.46 (m, 5 H, Ar*H*), 7.11−7.35 (m, 4 H, Ar*H*), 6.97 (s, 1 H, Ar*H*), 4.64 (s, 2 H, SCH_2), 4.08 (q, ArCH$_2$CH_2, J = 6.0 Hz), 3.12 (t, 2 H, ArCH_2, J = 6.0 Hz), and signals due to a minor rotamer (ca. 25%) at 4.77 (s), 3.78 (q, J = 6.0 Hz). ^{13}C NMR ($CDCl_3$): δ 197.3, 136.6, 134.2, 133.5, 132.9, 128.7, 128.0, 127.9, 127.3, 127.2, 126.5, 126.2, 122.6, 122.4, 119.9, 118.9, 112.5, 111.5, 47.4, 40.3, 24.1, 1.2 and signals due to a minor rotamer (ca. 20%) at δ 46.0, 42.0. IR (KBr) ν_{max} cm^{-1}: 3436, 3191, 2914, 2837, 1592, 1515, 1451, 1387, 1358, 1326, 1300, 1204, 1089, 999, 935, 816, 736. EI-MS: *m*/*z* (%): 130 (100), 202 (24). GC t_R = 14.7 min. NP-HPLC t_R = 5.7 min. RP-HPLC t_R = 13.2 min.

N-[2-(Indol-3-yl)ethyl]-S-[(pyrid-3-yl)methyl]dithiocarbamate (17). The general method was used with tryptamine as the amine and CH_2Cl_2 as the solvent. 3-(Bromomethyl)pyridine, HBr salt, was used as the alkylating agent in place of iodomethane, and 2.0 equiv of Et_3N were used. The crude product was chromatographed with EtOAc/hexanes (3:1) to afford a powdery tan solid (21% yield); mp = °C. ^{1}H NMR ($CDCl_3$): 8.5 (m, 2 H, Ar*H*), δ 8.16 (br s, 1 H), 7.69 (m, 1 H, Ar*H*), 7.58 (t, 1 H, Ar*H*, J = 6.0 Hz), 7.37 (d, 1 H, Ar*H*, J = 6.0 Hz), 7.24−7.12 (m, 4 H, Ar*H*), 7.03 (m, 1 H, Ar*H*), 4.52 (s, 2 H, SCH_2), 4.08 (m, 2 H, ArCH$_2$CH_2), 3.12 (m, 2H, ArCH_2), and signals due to a minor rotamer (ca. 25%) at 4.58 (s), 3.75 (m). ^{13}C NMR ($CDCl_3$): δ 197.0, 150.3, 148.8, 136.8, 133, 127, 123.6, 122.7, 122.4, 120, 118.9, 112.5, 111.6, 53.5, 47.7, 36.9, 24.2 and a signal due to a minor rotamer at 54.0. IR (KBr) ν_{max} cm^{-1}: 3403, 3306, 3164, 2917, 1724, 1619, 1500, 1455, 1421, 1392, 1332, 1257, 1089, 926, 851, 739. EI-MS: *m*/*z* (%): 130 (100), 202 (35). GC t_R = 14.8 min. NP-HPLC t_R = 27.9 min. RP-HPLC t_R = 9.4 min.

N-[2-(Indol-3-yl)ethyl]-S-[(pyrid-4-yl)methyl]dithiocarbamate (18). The general method was used with tryptamine as the amine and CH_2Cl_2 as the solvent. 4-(Bromomethyl)pyridine, HBr salt, was used as the alkylating agent in place of iodomethane, and 2.0 equiv of Et_3N was used. The crude product was recrystallized with EtOAc/hexanes (3:1) to afford tan crystals (50% yield); mp = 125−127 °C. ^{1}H NMR ($CDCl_3$): 8.51 (m, 2 H, Ar*H*), 8.08 (br s, 1 H), 7.59 (m, 1 H, Ar*H*), 7.39 (d, 1 H, Ar*H*, J = 6.9 Hz), 7.28−7.00 (m, 6 H, Ar*H*), 4.51 (s, 2 H, SCH_2), 4.07 (m, ArCH$_2$CH_2, J = 6.0 Hz), 3.14 (m, 2 H, ArCH_2), and signals due to a minor rotamer (ca. 25%) at 4.60 (s), 3.75 (m). ^{13}C NMR ($CDCl_3$): δ 196.3, 150.1, 146.7, 136.7, 127.4, 124.3, 124.1, 122.7, 122.4, 120.0, 118.9, 112.5, 111.6, 47.9, 38.5, 24.2, 19.8. IR (KBr) ν_{max} cm^{-1}: 3404, 3299, 2917, 2851, 2178, 2099, 1600, 1508, 1455, 1416, 1337, 1225, 1091, 1002, 927, 743. EI-MS: *m*/*z* (%): 130 (100), 202 (29). GC t_R = 14.7 min. NP-HPLC t_R = 28.9 min. RP-HPLC t_R = 9.4 min.

General Method for the Synthesis of Thioureas. The amine was dissolved/suspended in CH_2Cl_2, cooled to 0 °C, and treated with Et_3N (2.1−2.2 equiv). Methyl isothiocyanate (1.1−1.5 equiv) was added about 5 min later, and the reaction was allowed to slowly warm to room temperature while stirring overnight.

N-[1-(Indol-3-yl)methyl]-N′-methyl-thiourea (19). The general method was used with **25**. The volatiles were removed from the reaction, and the crude residue was recrystallized from EtOAc/hexanes to yield a gold, crystalline solid (54% yield); mp 148−150 °C. ^{1}H NMR (DMSO-d_6): δ 10.9 (br s, 1H, N*H*), 7.65 (m, 1H, Ar*H*), 7.36 (m, 2H, Ar*H*), 7.10 (t, 1H, Ar*H*, J = 7.2 Hz), 4.75 (br s, 2H, ArCH_2), 2.85 (br s, 3H, NHCH_3). ^{13}C NMR (DMSO-d_6): δ 183.4, 137.1, 124.9, 124.4, 122.1, 119.4, 112.7, 111.9, 40.1 (overlapped with *C*DCl$_3$), 31.5. IR (KBr) ν_{max} cm^{-1}: 3210, 1565, 1456, 1300, 1089. NP-HPLC (isocratic) t_R = 23.9 min. NP-HPLC (gradient) t_R = 22.5 min.

N-[1-(Indol-3-yl)ethyl]-N′-methyl-thiourea (20). The general method was used with tryptamine HCl. The crude product was isolated by washing the reaction mixture with 1 M H_2SO_4 (2×), saturated $NaHCO_3$, and brine and drying with Na_2SO_4. After concentration, the crude product was further purified by chromatography with EtOAc/hexanes (gradient, 1/1 to 3/1) to afford an oil that crystallizes on sitting to a light brown solid (92% yield); mp = 102−106 °C. ^{1}H NMR ($CDCl_3$): δ 8.13 (s, 1H, N*H*), 7.60 (d, 1H, Ar*H*, J = 7.8 Hz), 7.37 (d, 1H, Ar*H*, J = 8.1 Hz), 7.21 (t, 1H, Ar*H*, J = 7.0 Hz), 7.12 (t, 1H, Ar*H*, J = 7.0 Hz), 7.04 (s, 1H, Ar*H*), 5.75 (br s, 2H, N*H*-C=S), 3.79 (br d, 2H, ArCH$_2$CH_2, J = 5.4 Hz), 3.06 (t, 2H, ArCH_2, J = 6.6 Hz), 2.79 (br d, 3H, CH_3, J = 4.5 Hz). ^{13}C NMR ($CDCl_3$): δ 182.3, 136.3, 127.1, 122.4, 122.3, 119.6, 118.5, 112.4, 111.4, 44.8, 30.5, 24.8. IR (KBr) ν_{max} cm^{-1}: 3394, 3320, 3323, 3051, 1561, 1342. NP-HPLC t_R = 24.2 min. RP-HPLC (1/1 MeOH/H_2O) t_R = 6.1 min.

Brassitin (21).[29] Freshly made **25** (190 mg, 1.3 mmol) and Et_3N (271 μL, 1.95 mmol) were dissolved in anhydrous MeOH (10 mL). The flask was cooled to 0 °C, and methyl chlorothiolformate (116 μL, 1.36 mmol) was added dropwise followed by stirring at room temperature for 6 h. A few drops of H_2O were added to quench excess reagent, and the volatiles were evaporated. The residue was dissolved in EtOAc (35 mL) and washed with 0.5 M HCl (2 × 20 mL), saturated $NaHCO_3$ (20 mL), and brine (15 mL). The organic solution was dried with Na_2SO_4, filtered, and concentrated to afford a crude brownish-orange solid (270 mg). After recrystallization from CH_2Cl_2/hexanes, beige crystals: 125 mg, 44% yield; mp 110−111 °C. ^{1}H NMR ($CDCl_3$): δ 8.15 (br s, 1H, N*H*), 7.64 (d, 1H, Ar*H* J = 7.9), 7.39 (d, 1H, Ar*H* J = 7.1), 7.23 (m, 1H, Ar*H*), 7.18 (m, 1H, Ar*H*), 7.13 (m, 1H, Ar*H*), 5.52 (br s, 1H, CH_2N*H*C), 4.67 (d, 2H, ArC*H*$_2$, J = 5.1), 2.38 (s, 3H, SC*H*$_3$). ^{13}C NMR ($CDCl_3$): δ 167.6, 136.3, 126.3, 123.3, 122.5, 119.9, 118.7, 112.1, 111.3, 36.9, 12.4. EI-MS *m*/*z* (%): 220 (37, M^+), 205 (9), 172 (12, M^+-SCH_3), 130 (100). NP-HPLC t_R = 9.8 min. RP-HPLC (1/1 CH_3CN/H_2O + 0.1% TFA) t_R = 9.5 min.

N-[(Indol-3-yl)methyl]propanamide (29). Compound **25** (1.00 g, 6.84 mmol) and Et_3N (1.4 mL, 10.26 mmol) were dissolved in MeOH and cooled to 0 °C. Propionyl chloride (633 mg, 6.84 mmol) was added dropwise, and the reaction was stirred at room temperature for 4 h. The volatiles were removed, and the residue was taken up in CH_2Cl_2 (40 mL), washed with 10% citric acid (20 mL), saturated $NaHCO_3$ (20 mL), and brine (20 mL). The organic layer was dried with Na_2SO_4 and filtered, and the volatiles were removed to yield 1.33 g of a white, crystalline solid (1.33 g, 96% yield). An analytical sample was recrystallized form EtOAc/hexanes; mp 91−92 °C. ^{1}H NMR ($CDCl_3$): δ 8.80 (br s, 1H, N*H*), 7.62 (d, 1H, Ar*H*, J = 7.85 Hz), 7.38 (d, 1H, Ar*H*, J = 7.2 Hz), 7.20 (3H, Ar*H*), 5.80 (br s, 1H, N*H*), 4.60 (d, 2H, ArC*H*$_2$, J = 5.1 Hz), 2.20 (q, 2H, COC*H*$_2$CH$_3$, J = 7.6 Hz), 1.14 (t, 3H, COCH$_2$C*H*$_3$, J = 7.6 Hz). ^{13}C NMR ($CDCl_3$): δ 173.6, 136.5, 126.6, 123.3, 122.5, 119.9, 118.8, 112.8, 111.4, 35.2, 29.7, 9.9. IR (KBr) ν_{max} cm^{-1}: 3405, 1891, 1634, 1532, 1097.

N-[(Indol-3-yl)methyl]propanethioamide (22). Amide **29** (190 mg, 0.94 mmol) was dissolved in THF (20 mL). Lawesson reagent (304 mg, 0.75 mmol) was added to the resulting solution, and the reaction was stirred for 2 h at room temperature. The volatiles were removed, and the residue was dissolved in CH_2Cl_2 (20 mL) and washed with H_2O (12 mL). The organic layer was dried with Na_2SO_4 and filtered. After standing, a white precipitate formed, which was filtered, and the filtrate was concentrated. The resulting residue (380 mg) was chromatographed with EtOAc/hexanes (1:1) to yield a clear oil, which slowly crystallized (85 mg, 41% yield); mp 132−134 °C. ^{1}H NMR ($CDCl_3$): δ 8.23 (br s, 1H, N*H*), 7.63 (1H, Ar*H*), 7.41 (1H, Ar*H*), 7.23 (3H, Ar*H*), 4.98 (d, 2H, ArC*H*$_2$, J = 4.5 Hz), 2.68 (q, 2H, CSC*H*$_2$CH$_3$, J = 7.5 Hz), 1.30 (t, 3H, CSCH$_2$C*H*$_3$, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$): δ 205.9, 136.3, 126.5, 124.0, 122.8, 120.3, 118.7, 111.5, 110.8, 42.2, 40.0, 13.5. IR (KBr) ν_{max} cm^{-1}: 3331, 2975, 2931, 1523, 1413, 1090. EI-MS *m*/*z* (%): 218 (49, M^+), 163 (8), 131 (12), 130 (100). NP-HPLC t_R = 10.9 min. RP-HPLC t_R = 10.4 min.

2-Naphthoyl Chloride. A 100 mL round-bottom flask was charged with 2-naphthoic acid (2 g, 11.6 mmol) and $SOCl_2$ (15 mL). The solution was refluxed for 4 h and then concentrated to yield a yellow solid, which was used without further purification (2.21 g, 100% yield). ^{1}H NMR ($CDCl_3$): δ 8.76 (s, 1H, Ar*H*), 8.04 (2H, Ar*H*), 7.93 (d, 2H, Ar*H*, J = 8.9 Hz), 7.66 (m, 2H, Ar*H*).

2-Naphthamide (27). 2-Naphthoyl chloride (2.21 g, 11.6 mmol) was dissolved in a MeOH/NH_3 solution (2 M, 20 mL) and was allowed to stir overnight. Volatiles were removed, and the resulting white solid was triturated with EtOAc. The solid was filtered and washed with cold EtOAc to yield a white solid, which was used without further purification (1.98 g, 100% yield); mp 191−192 °C. ^{1}H NMR ($CDCl_3$): δ 8.39 (s, 1H, Ar*H*), 7.90 (4H, Ar*H*), 7.57 (m, 2H, Ar*H*). ^{13}C NMR ($CDCl_3$): δ 169.3, 135.0, 132.6, 130.5, 129.0, 128.6, 128.1, 127.9, 127.8, 126.9, 123.7. IR (Nujol) ν_{max} cm^{-1}: 3400, 3210, 1650, 1628, 1512, 1510.

2-Aminomethylnaphthalene (28). Compound **27** (1.00 g, 5.8 mmol) in THF (20 mL) was added slowly to a solution of LAH (1.76 g, 46.4 mmol) in THF (45 mL) at 0 °C. The solution was allowed to warm to room temperature, and the reaction was stirred overnight. The reaction was cooled to 0 °C and quenched with H_2O. The solids were filtered from the solution through Celite and washed with hot THF. The filtrate was concentrated, and the residue was dissolved in EtOAc (80 mL) and washed with 1 M HCl (3 × 30 mL). The aqueous layer was basified with 6 M NaOH to a pH of 12, and the precipitate was extracted with EtOAc (3 × 30 mL). The resulting organic solution was washed with brine (40 mL), dried with Na_2SO_4, and filtered. Concentration afforded a slightly yellow solid (510 mg, 56% yield); mp 55−56 °C. ^{1}H NMR ($CDCl_3$): δ 7.80 (3H, Ar*H*), 7.72 (s, 1H, Ar*H*), 7.43 (m, 3H, Ar*H*), 4.00 (s, 2H, ArC*H*$_2$). ^{13}C NMR ($CDCl_3$): δ 140.6, 133.5, 132.5, 128.2, 127.7, 126.1, 125.8, 125.5, 125.1, 46.6. IR (KBr) ν_{max} cm^{-1}: 3362, 3291, 3050, 2915, 1950, 1596, 1507, 1358, 1273. GC t_R = 9.0 min. EI-MS *m*/*z* (%): 157 (83, M^+), 156 (100), 141 (15), 129 (49), 128 (40), 127 (24), 115 (10).

1-Bromo-3-(indol-3-yl)propanone (31). The diazoketone **30** (379 mg, 1.90 mmol) was dissolved in acetic acid (4 mL) and cooled to 0 °C. HBr (48%, 0.51 mL) was added dropwise. Forty minutes later, the reaction was diluted with H_2O and then quenched at 5 °C with saturated $NaHCO_3$. The reaction mixture was extracted with CH_2Cl_2 (2×), washed with saturated $NaHCO_3$, H_2O, and brine, dried with Na_2SO_4, filtered, and concentrated to a brown oil (398 mg, 83% yield). The crude product was used immediately in the next step. ^{1}H NMR ($CDCl_3$): δ 8.26 (br s, 1H, N*H*), 7.56 (d, 1H, Ar*H*, J = 7.8 Hz), 7.39 (d, 1H, Ar*H*, J = 7.8 Hz), 7.27−7.13 (m, 3H, Ar*H*), 4.07 (s, 2H, C*H*$_2$Br), 3.95 (s, 2H, ArC*H*$_2$).

General Method for the Synthesis of Thiazoles. α-Bromoketone **31** was dissolved in EtOH and treated with thioamide (1.5 equiv) and $NaHCO_3$ (1.5 equiv). The resulting mixture was heated at reflux overnight. Upon cooling, the reaction material was partitioned between EtOAc and half saturated $NaHCO_3$. The aqueous layer was extracted with EtOAc, and the combined organic layers were washed with H_2O and brine, dried with $MgSO_4$, filtered, and concentrated to a brown oily solid. The crude thiazole product was purified by chromatography with EtOAc/hexanes (1:2).

4-[(Indol-3-yl)methyl]thiazole (23). The general method was used with thioformamide[30] to afford a 71% yield. ^{1}H NMR ($CDCl_3$): δ 8.76 (d, 1H, SC*H*N, J = 2.0 Hz), 8.11 (br s, 1H, N*H*), 7.52 (d, 1H, Ar*H*, J = 7.6 Hz), 7.36 (d, 1H, Ar*H*, J = 8.0 Hz), 7.18 (t, 1H, Ar*H*, J = 7.1 Hz), 7.08 (t, 2H, Ar*H*, J = 7.4 Hz), 6.90 (s, 1H, Ar*H*), 4.34 (s, 2H, ArC*H*$_2$). ^{13}C NMR ($CDCl_3$): δ 157.5, 152.5, 122.5, 122.1, 119.4, 119.1, 113.8, 113.5, 111.2, 27.6. IR (CH_2Cl_2) ν_{max} cm^{-1}: 3626, 3470, 3051, 2987, 1420, 1264. GC t_R = 15.1 min. EI-MS *m*/*z* (%): 214 (100, M^+), 213 (86), 186 (15), 154 (14), 130 (51). RP-HPLC (1/1 CH_3CN/H_2O + 0.1% TFA) t_R = 4.4 min.

4-[(Indol-3-yl)methyl]-2-methyl-thiazole (24). The general method was used with thioacetamide to afford a 56% yield. ^{1}H NMR ($CDCl_3$): δ 8.17 (br s, 1H, N*H*), 7.53 (d, 1H, Ar*H*, J = 7.8 Hz), 7.34 (d, 1H, Ar*H*, J = 8.1 Hz), 7.17 (t, 1H, Ar*H*, J = 7.0 Hz), 7.10−7.04 (m, 2H, Ar*H*), 6.63 (s, 1H, Ar*H*), 4.23 (s, 2H, C*H*$_2$), 2.69 (s, 3H, C*H*$_3$). ^{13}C NMR ($CDCl_3$): δ 165.6, 162.3, 156.1, 136.4, 127.3, 122.6, 122.0, 119.3, 119.1, 113.4, 111.1, 27.7, 19.1. IR (KBr) ν_{max} cm^{-1}: 3247, 3090, 2919, 1527, 1454, 1429, 1188. GC t_R = 15.4 min. EI-MS *m*/*z* (%): 228 (100, M^+), 227 (71), 186 (22), 154 (23), 130 (39). RP-HPLC (1/1 MeOH/H_2O) t_R = 18.6 min.

Computational Procedure. All electronic structure calculations were carried out using the Gaussian 03 suite of programs.[31] Natural bond orbital (NBO) population analysis was done with NBO 3.1 as implemented in Gaussian 03.[32] All compounds with terminal methyl groups were optimized at the HF/6-31G*//HF/6-31G[33] level. ESP[34] and NBO atomic charges were computed. The HF/6-31G* molecular ESP surface was mapped onto the total density surface.

Inhibition Assays with IDO. The inhibition assays were performed in a 96 well microtiter plate as described by Littlejohn et al.[14] with a small modification. Briefly, the reaction mixture contained 50 mM potassium phosphate buffer (pH 6.5), 40 mM

ascorbic acid, 400 μg/mL catalase, 20 μM methylene blue, and purified recombinant IDO(1) optimized based on its activity. The reaction mixture was added to the substrate, L-Trp, and the inhibitor. The L-Trp was serially diluted from 200 to 25 μM, and the inhibitors were tested at two concentrations, 200 and 400 μM. The reaction was carried out at 37 °C for 60 min and stopped by adding 30% (w/v) trichloroacetic acid. The plate was heated at 65 °C for 15 min to convert formylkynurenine to kynurenine and then was spun at 6000*g* for 5 min. Finally, 100 μL of supernatant from each well was transferred to a new 96 well plate and mixed with 2% (w/v) *p*-(dimethylamino)benzaldehyde in acetic acid. The yellow color generated from the reaction with kynurenine was measured at 490 nm using a Synergy HT microtiter plate reader (Bio-Tek, Winooski, VT). The data were analyzed using Graph Pad Prism 4 software (Graph Pad Software Inc., San Diego, CA).

Acknowledgment. We thank the National Institutes of Health (NCI R01-CA 109542) and Bryn Mawr College (Faculty Research Fund) for financial support of this work. We are grateful to Prof. Michelle Francl of Bryn Mawr College for assistance with the computational experimentation.

Supporting Information Available: Copies of ^{1}H NMR spectra for compounds **1**−**24**, **27**−**29**, and **31**. Copies of ^{13}C NMR spectra for compounds **1**−**24** and **27**−**29**. Copies of HPLC data for compounds **1**−**24**. Copies of GC data for compounds **15**, **17**, **18**, **23**, **24**, and **28**. Copies of MS data for compounds **15**, **17**, **18**, **23**, **24**, and **28**. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0508888